MECHANISMS OF RESISTANCE TO β-LACTAM ANTIBIOTICS IN *BACTEROIDES* SPECIES

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CONTENTS

CHAPTER 2

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ABSTRACT

Mechanisms responsible for resistance to β-lactam antibiotics were investigated in clinical isolates of *Bacteroides* spp., the most common anaerobic Gram negative pathogen. Among 108 isolates of *Bacteroides* spp. obtained from clinical material in Nottingham, 69 (64%) were identified as *Bacteroides fragilis*. Approximately one-fifth of the *Bacteroides* spp. produced elevated levels of β-lactamases, and many of these strains showed increased resistance to β-lactam antibiotics usually regarded as β-lactamase stable.

Four β-lactamase types were identified: Type one was represented by zinc dependent metallo-β-lactamases that hydrolysed cefoxitin, latamoxef and imipenem. Type two displayed intermediate to high specific activity in tests with nitrocefin as substrate and hydrolysed cefoxitin and latamoxef, but not imipenem. Type three exhibited intermediate levels of specific activity and caused reduced susceptibility to cefoxitin, latamoxef and imipenem, although they hydrolysed these antibiotics inefficiently. Type four probably represented enhanced production of the β-lactamases characteristic of most bacteroides strains. Organisms producing this enzyme normally remained susceptible to cefoxitin, latamoxef and imipenem, and the enzyme was fully susceptible to β-lactamase inhibitors, including clavulanic acid.

Several strains were detected that exhibited reduced susceptibility to imipenem that was not related to metallo-β-lactamases production. In contrast, one strain that produced a metallo-β-lactamase remained fully sensitive to imipenem as judged by conventional titration. Investigation of isolates producing metallo-β-lactamase and several similar ones encountered in an earlier study showed a correlation between the degree of resistance to imipenem and specific imipenemase activity.

In an attempt to elucidate reduced susceptibilty to imipenem that was not related to metallo-β-lactamase production, cell envelope properties and penicillin-binding proteins (PBPs) of selected strains

were investigated. Studies of outer membrane proteins and lipopolysaccharide composition of *B.fragilis* strains unexpectedly showed that metallo-β-lactamase producers displayed unusual cell envelope profiles. Those strains that showed reduced susceptibility that was not associated with β-lactamase appeared to be normal, although two of these strains displayed abnormally high crypticity values.

Between three and six PBPs were visualised in tests with ^{3}H-benzylpenicillin. PBPs 1 to 3 were present in all strains, but were observed in imipenem-resistant strains only when tests were carried out in the presence of β-lactamase inhibitors. Competitive binding experiments indicated that imipenem showed affinity for the high molecular weight PBPs of sensitive *B.fragilis*. The low molecular weight PBPs 4 to 6 were detected intermittently and only in certain strains, notably those that exhibited reduced susceptibility to imipenem. PBP 6 was found only in strains showing non-enzymic resistance.

The results suggest that several different types of resistance to β-lactam antibiotics are circulating in clinical isolates of *Bacteroides* spp. in Nottingham: firstly, strains that produce a metallo-β-lactamase that hydrolyses 'β-lactamase-stable' compounds, including imipenem; secondly, strains producing enzymes that hydrolyse cefoxitin and latamoxef, but not imipenem; thirdly, strains that possess a permeability barrier that affects imipenem as well as other β-lactam antibiotics; fourthly, strains with altered penicillin-binding proteins; and fifthly strains that produce raised amounts of 'normal' β-lactamase. The latter strains slowly hydrolyse imipenem and other β-lactam compounds and this may be a factor in the reduced susceptibility to these agents.

Clinical isolates that possess these resistance mechanisms may appear susceptible according to conventional break-point criteria *in vitro*. Never the less, they exhibit considerably reduced susceptibility to β-lactam agents and the therapeutic implications of this need investigating. At present such strains represent a relatively small proportion of clinical isolates of *Bacteroides* spp., but the prevalence of resistance needs to be carefully monitored.

CHAPTER 1

INTRODUCTION

1:1 General Introduction

1:2 Classification and characterisation of the genus *Bacteroides*

1:3 Clinical importance and pathogenicity of *Bacteroides* species

1:4 Treatment of infections due to *Bacteroides* species

1:5 β-lactam antibiotics: their characterisation, classification and spectrum of activity

1:6 In-vitro susceptibility of *Bacteroides* species to β-lactams and other antibiotics

1:7 Penetration of the antibiotic into the bacterial cell

1:8 Mode of action of β-lactam antibiotics

1:9 Mechanisms of resistance to β-lactam antibiotics

1:10 Inactivation of the antibiotic by enzymic attack

1:11 Classification of β-lactamases

1:12 β-Lactamases of *Bacteroides* species

1:13 Reduction of penetration of the antibiotic

1:14 Alteration of penicillin binding proteins

1:15 Aims of study

CHAPTER 1

INTRODUCTION

1:1 General Introduction

Improvements in laboratory techniques for the isolation, cultivation and identification of anaerobic bacteria has heightened awareness of the role of obligate anaerobes in general, and *Bacteroides* species in particular, in a wide variety of infections. Many of the anaerobic bacteria isolated from an infected site are considered to be opportunistic pathogens and are often part of a mixed infection (McGowan and Gorbach, 1981).

In the UK, the two main groups of antimicrobial agents employed to combat these infections are the β-lactam antibiotics and the nitro-imidazoles, particularly metronidazole. Metronidazole is effective against virtually all obligate anaerobes but not against the other bacteria which may be present in polymicrobial infections. β-Lactam antibiotics are active against a wide range of bacterial species, including many anaerobic species, but resistance among the bacteroides is relatively common (Phillips *et al.*, 1981). β-Lactamase stable β-lactam antibiotics have improved activity against *Bacteroides* species, but problems of resistance are still apparent (Betriu *et al.*, 1992; Phillips *et al.*, 1992).

In this study, an investigation of the resistance of *Bacteroides* species to β-lactam antibiotics was carried out, with detailed examination of bacteroides β-lactamases. Mechanisms of resistance to carbapenems among *B.fragilis* isolates were explored with consideration of carbapenemase activity, permeability barriers and penicillin binding proteins.

1:2 Classification and characterisation of the genus *Bacteroides*

Until recently the genus *Bacteroides* was represented by a heterogeneous group of Gram negative, obligately anaerobic, non sporing bacilli which, together with the genera *Fusobacterium* and *Leptotrichia*, belonged to the family Bacteroidaceae. The species of bacteroides were divided into 5 main groups: the fragilis group; the melaninogenicus-oralis group; an asaccharolytic group; rumen species; and 'others' (Duerden, 1990). However, lack of good criteria for defining the genus, including the unacceptably wide range of DNA base composition of G+C (28-61 mol per cent), led to a large accumulation of more than 50 heterogeneous species (Holderman *et al.*, 1984).

To resolve this unsatisfactory position, Shah and Collins (1989) made proposals restricting the genus *Bacteroides* to saccharolytic, non-pigmented species whose DNA base composition is within the range G+C 40-48 mol per cent and which are biochemically and chemically related to the type species *Bacteroides fragilis*. By these criteria, the genus *Bacteroides* is now comprised of only 10 species, including *B.fragilis, B.distasonis, B.ovatus, B.thetaiotaomicron and B.vulgatus*, all members of the previously named 'fragilis group'. These species produce acetate and succinate as major metabolic end products, have enzymes of the pentose phosphate pathway such as glucose-6-phosphate dehydrogenase (G-6-PDH) and 6-phosphogluconate dehydrogenase (6-PGDH), and contain a mixture of long chain fatty acids with predominantly straight chain saturated, anteiso-methyl branched and iso-methyl branched acids. The growth of these species is stimulated by bile.

The moderately saccharolytic species represented by the

melaninogenicus-oralis group have been reclassified in a new genus, *Prevotella* (Shah and Collins, 1990). These species differ from the *B.fragilis* group in that they are sensitive to bile and lack the enzymes G-6-PDH and 6-PGDH. They have a DNA G+C composition between 40 and 50 mol per cent, are largely indigenous to the oral cavity and produce mainly acetic and succinic acids in a glucose medium. The 16 members of the *Prevotella* genus include species previously named *B.bivius, B.buccae, B.buccalis, B. disiens and B.oralis,* and the pigmented species *B.melaninogenicus* and *B.intermedius* which produce both protohaem and protoporphyrin pigments.

The asaccharolytic pigmented species, *B.asaccharolyticus, B.gingivalis and B.endodontalis*, comprise another tight cluster that differs so significantly from the new genera *Bacteroides* and *Prevotella* that they have been reclassified in a new genus, *Porphyromonas* (Shah and Collins, 1988). These species produce major amounts of protohaem on blood agar plates, lack G-6-PDH and 6-PGDH, produce high levels of butyric acid and possess predominantly isomethyl branched long chain fatty acids.

The taxonomic position for many other species, such as *B.ureolyticus*, *B.forsythus* and *B.gracilis,* remains uncertain. The taxonomic revision of clinically important species previously included in the genus *Bacteroides* is summarised in Table 1.1.

1:3 Clinical importance and pathogenicity of *Bacteroides* species

The pathogenic potential of anaerobic Gram negative, non-sporing, anaerobic bacilli has been recognised since the late nineteenth century (Veillon and Zuber, 1897). Many early reports

Table 1.1 Current taxonomic status of clinically important species previously included in the genus *Bacteroides*.

Group/Species	Current genus name
Saccharolytic group	
(*B.fragilis* group)	*Bacteroides*
B.fragilis, B.caccae, B.distasonis.	
B.eggerthii, B.merdae, B.ovatus, B.stercoris	
B.thetaiotaomicron, B.uniformis, B.vulgatus	
Moderately saccharolytic group	
(*B.oralis-B.melaninogenicus* group)	*Prevotella*
B.melaninogenicus, B.bivius, B.buccae,	
B.buccalis, B.corporis, B.denticola, B.disiens,	
B.heparinolyticus, B.intermedius, B.loescheii,	
B.oralis, B.oris, B.oulorum, B.ruminicola,	
B.veroralis, B.zoogleoformans	
Pigmented asaccharolytic group	*Porphyromonas*
B.asaccharolyticus, B.gingivalis,	
B.endodontalis	
Others (uncertain taxonomic status)	
B.levii, B.macacae	Possibly *Porphyromonas*
B.ureolyticus	*Campylobacter* or *Wolinella*
B.gracilis	Possibly *Wolinella*
B.capillosus	Probably new genus
B.splanchnicus	Probably new genus
B.forsythus	Probably new genus

From Shah and Gharbia (1991)

described low isolation rates of anaerobes. Dack (1940) isolated non-sporing anaerobes from less than 4% of specimens from patients in a surgical unit and Stokes (1958) found anaerobes in 10% of over 4000 specimens in which bacteria were present. These figures probably underestimate the incidence of anaerobic infections because of the poor anaerobic techniques used at that time. The impact of good anaerobic culturing methods was highlighted by Leigh (1974) who showed that the isolation rate of *Bacteroides* species from wound infections after abdominal surgery rose from 17% to 81% as a result of improved anaerobic techniques during a period when the rate of postoperative infection was constant.

Bacteroides species are an important cause of purulent infections at body sites adjacent to colonized mucosal surfaces. Holland *et al.* (1977) found 70% of the anaerobes isolated from clinical samples, excluding those from stool, urine, sputum and blood, were identified as *Bacteroides* species. The *B.fragilis* group account for most of the 'bacteroides' (old nomenclature) isolated from infected sites, with *B.fragilis* the predominant species (Duerden, 1980a).

The most common bacteroides infections are intraabdominal abscesses, peritonitis and wound infections associated with the appendix or large intestine (Leigh, 1974). Finegold (1977) stated that *Bacteroides* species of the fragilis group were associated with 86% of intraabdominal infections. *Bacteroides* species are also principal pathogens of the female genital tract and are present in two thirds of the cases of pelvic abscesses (Gorbach and Bartlett, 1974; Thadapalli *et al.*, 1973). Anaerobic pleuropulmonary infections include lung abscesses, necrotizing pneumonia and empyema and *B.fragilis* is isolated in 15% of these cases (Bartlett and Finegold, 1974). Gram negative anaerobic bacilli are the commonest cause of brain

abscesses, *B.fragilis* being most frequently implicated in those secondary to otitis media (Ingham *et al.*, 1977b). *B.fragilis* can also be isolated from skin and soft-tissue infections, and is a major pathogen in cases of diabetic foot ulcer (Louie *et al.*, 1976). Bacteraemia is commonly associated with *B.fragilis* infections, but endocarditis due to *Bacteroides* species is rare.

Certain underlying conditions, such as impairment of the blood supply, disruption of the mucosal barriers or tissue destruction can predispose to anaerobic infection. A preceding infection by an anaerobic or facultative organism may also predispose a site to invasion by the indigenous anaerobic flora. Anaerobic bacteria involved in infections are usually derived from the normal flora of the skin, oral cavity, nasopharynx, large intestine or female genital tract. The infecting flora is complex, and therefore infections are usually polymicrobial and often pyogenic (McGowan and Gorbach, 1981).

In these endogenous infections, the bacterial species present do not reflect the composition of the normal flora at the site of infection (Duerden, 1980a,b). Infections with *B.fragilis* usually originate from the lower gastrointestinal tract. The numerically dominant *Bacteroides* species in the normal colonic flora are *B.distasonis, B.vulgatus* and *B.thetaiotaomicron*, whereas *B.fragilis* accounts for only 5% of the colonic microflora (McGowan and Gorbach, 1981). *B.fragilis*, however, is more commonly associated with intra-abdominal and soft tissue infections than are the other *Bacteroides* species. Also, *B.fragilis* is responsible for a large proportion of serious bacteroides infections, such as bacteraemia, wound infections, intraperitoneal abscesses and peritonitis (Polk and Kasper, 1977; Duerden, 1980a).

These observations suggest that *B.fragilis* has unique

virulence properties. Kasper (1976) showed the presence of capsules in *B.fragilis* by electron microscopy. These polysaccharide capsules are an important factor for virulence and abscess formation in experimental animals (Onderdonk *et al.*, 1977, 1984; Brook, 1987). The capsule has been shown to be associated with resistance to phagocytosis and intracellular killing by polymorphonuclear leucocytes, suppression of macrophage phagocytosis, and adherence to peritoneal surfaces (Reid and Patrick, 1984; Rodloff *et al.*, 1986; Onderdonk *et al.*, 1978). Adhesion of Gram negative bacteria to mucosal surfaces is generally associated with fimbriae. Van Doorn *et al.* (1987) isolated fimbriae from a strain of *B.fragilis* and Pruzzo *et al.* (1989) found a correlation between fimbriation and haemagglutination. Iron repressible outer membrane proteins have been demonstrated in *B.fragilis*, which may be important in the uptake of essential iron from the host (Otto *et al.*, 1990). *B.fragilis* produces extracellular or membrane-associated enzymes, including lipases, proteases and nucleases, which may cause the tissue damage and necrosis accompanying bacteroides infections (Rudek and Haque, 1976). The *B.fragilis* group is also able to tolerate exposure to oxygen, which is thought to be a prerequisite to virulence of anaerobes, by the production of superoxide dismutase and catalase (Tally *et al.*, 1977; Gregory *et al.*, 1977).

Furthermore, *B.fragilis* has been shown to protect facultatively anaerobic bacteria against phagocytosis and intracellular killing by reducing the opsonic capacity of serum or removal of essential serum components (Ingham *et al.*, 1977a, 1981; Jones and Gemmell, 1982). Therefore, apart from being an important pathogen, *B.fragilis* may also be a potentiator of infections caused by other organisms.

1:4 Treatment of infections due to *Bacteroides* species

Apart from antimicrobial therapy, surgical intervention, which includes drainage of abscesses and excision of necrotic tissue, is of great importance in the treatment of anaerobic infections. In some infections surgical therapy may be all that is required (Finegold, 1989). A single dose of a suitable antibiotic just before this surgical drainage has been reported to prevent bacteraemia and accelerate healing (Blick *et al.*, 1980).

When antibiotics are used to combat infections solely due to bacteroides or other anaerobes, metronidazole is the drug of choice. Metronidazole has very low toxicity and is consistently bactericidal for anaerobes. However, most infections involving *Bacteroides* species are polymicrobial and antibiotic therapy must be directed at both the anaerobic and aerobic components. In these situations, metronidazole is commonly used in combination with an anti - aerobe antibiotic, normally a cephalosporin or an aminoglycoside.

Another possible option in the treatment of infections due to bacteroides and aerobic pathogens is the use of single broad spectrum compounds. These would be simpler to administer and may be financially preferable. Candidates for such antibiotics include the cephamycins, carbapenems, latamoxef, and the combinations of penicillins with β-lactamase inhibitors. Cephamycins have been widely used, but are intrinsically less active than the carbapenems or the combination products. Latamoxef is generally well tolerated with a plasma elimination half-life of 2.5 hours and reasonably good penetration into serous fluids, but is associated with hypoprothrombinaemia in some patients (Lambert and O'Grady, 1992; Sanders and Aldridge, 1992). It is no longer marketed in the

UK.

The β-lactamases produced by *Bacteroides* species within an abscess cavity or infected tissue can protect other organisms in a mixed infection from the activity of susceptible β-lactam antibiotics. Brook and Yokum (1983) showed that the resistance of group A β-haemolytic streptococci to penicillin increased by 8,500-fold when they were mixed with cultures of *B.fragilis*. Also, Soriano *et al.* (1989) demonstrated the ability of a β-lactamase-producing strain of *B.fragilis* to shield *E.coli* from cefotaxime in peritoneal infections in rats.

Of the other antibiotics, clindamycin, although effective *in vitro* and *in vivo*, has lost its popularity because of an association with pseudomembranous colitis. Chloramphenicol, despite its broad antibacterial spectrum and good penetration into body fluids and tissues, is restricted to use in serious infections because of its potential for bone marrow toxicity.

Anaerobes, in particular *Bacteroides* species, play an important role in post - operative infections after abdominal surgery. To combat this, various prophylactic antibiotic regimens have been used. Metronidazole alone, or in combination with a cephalosporin or an aminoglycoside, has proved effective (Greenwood *et al*, 1991). Studies have also assessed the broad spectrum β-lactam antibiotics, which are active against bacteroides, as prophylactic agents and found them to be as efficient as metronidazole alone (Panichi *et al.*, 1982).

1:5 β-lactam antibiotics: their characterisation, classification and spectrum of activity

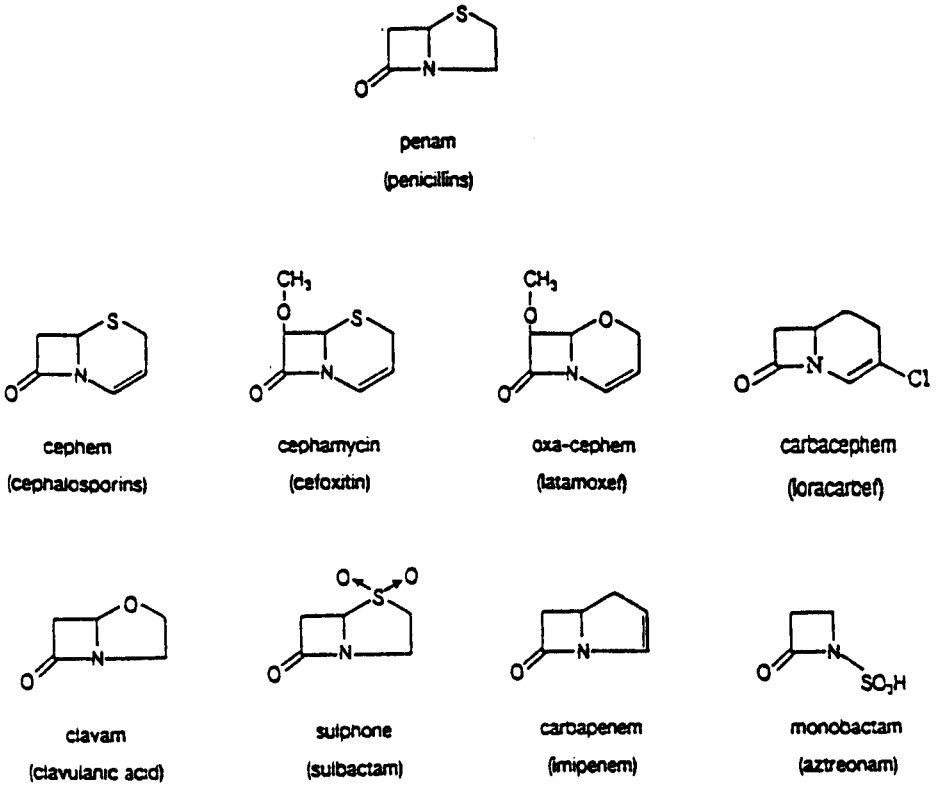
β-Lactam antibiotics are a large and therapeutically important

group of compounds which have in common the structural feature of a β-lactam ring. They can be divided, according to their chemical structure, into three broad categories: the penicillins or penams; the cephalosporins or cephems, with related cephamycins, oxa-cephems and carbacephems; and others including carbapenems, monobactams and the clavams and sulphones that are used as β-lactamase inhibitors.

Penicillins are N-acylated derivatives of 6-aminopenicillanic acid, comprising a β-lactam ring fused with a saturated 5-membered sulphur-containing thiazolidine ring. In cephalosporins, a six-membered dihydrothiazine ring containing sulphur and a double bond replaces the thiazolidine ring to give 7-aminocephalosporanic acid. The closely related cephamycins, for example cefoxitin, have an α-methoxy group substituted at the 7-position, and in oxacephems, such as latamoxef, the sulphur is replaced by oxygen. Substitution of the sulphur of cephalosporins with a carbon atom gives the corresponding carbacephem analogue, such as loracarbef. The remaining β-lactam compounds have a variety of ring structures or side chains attached to the β-lactam ring. Carbapenems, such as imipenem, differ in possessing a double bond in the thiazolidine ring and the substitution of CH_2 for sulphur. The only notable member of the clavams is clavulanic acid, the structure of which differs from penams by the substitution of oxygen for sulphur. The monobactams, such as aztreonam, are monocyclic; i.e. the β-lactam ring is not associated with a fused ring system. Sulbactam is an example of a penicillanic acid sulphone. The basic nuclear structures of the β-lactam family of antibiotics are given in Fig. 1.1.

The first member of this large group of compounds was

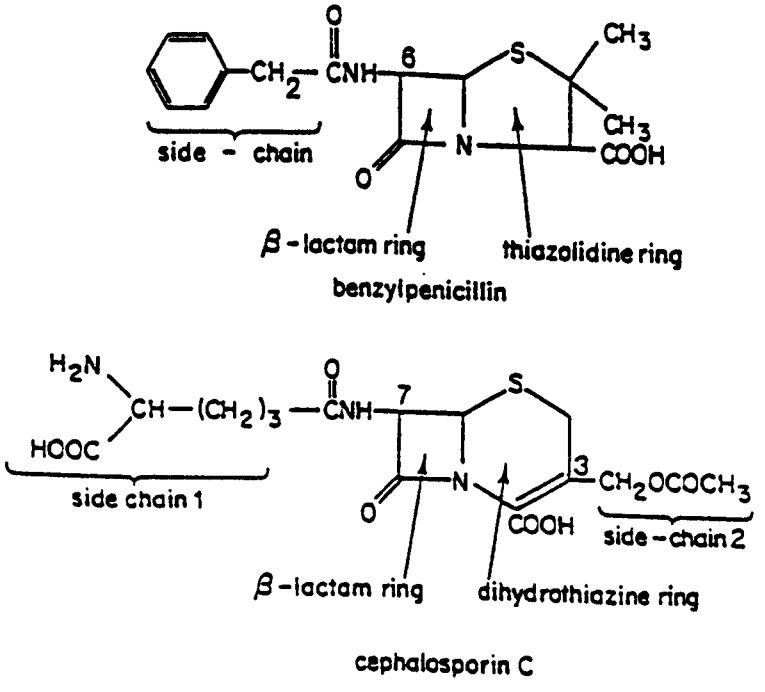
Fig. 1.1 Basic nuclear structures of β-lactam antibiotics currently available (examples in parentheses)



From Greenwood (1989) and Lees *et al.* (1993)

benzylpenicillin (penicillin G), which is produced by the mould *Penicillium chrysogenum*. Benzylpenicillin contains a phenylacetamido side chain at the 6-position of the fused ring system (Fig 1.2). It is active particularly against Gram positive organisms and Gram negative cocci. Streptococci, β-lactamase negative *Staphylococcus aureus* and *Neisseria* spp. are highly susceptible. The first improvements were made by inducing the producer organism to incorporate variant side chains into the penicillin molecule. By these means, phenoxymethylpenicillin (penicillin V) was produced; this exhibits enhanced acid stability and oral absorption without much loss of antibacterial activity. In the late 1950s, removal of the side chain of the benzylpenicillin nucleus was achieved by enzymatic cleavage, opening up the possibility of the development of semi-synthetic penicillins (Batchelor *et al.*, 1959). As a result, the antistaphylococcal penicillins, including methicillin and cloxacillin, were produced. These are resistant to breakdown by staphylococcal β-lactamases but display reduced antibacterial activity. In 1961, the spectrum of benzylpenicillin was extended to Gram negative bacilli by the addition of an amino group to the side-chain to form ampicillin which is not, however, β-lactamase stable (Rolinson and Stevens, 1961). A further modification in the spectrum of ampicillin was achieved by mecillinam, produced by introducing an amidino side chain linkage at the 6-position of the penicillanic acid nucleus (Lund and Tybring, 1972). A further change in spectrum to include antipseudomonal activity, is demonstrated by carbenicillin, in which a carboxyl group is substituted for the amino group of ampicillin (Knudsen *et al.*, 1967). This was improved by the thienyl variant of carbenicillin, ticarcillin (Neu and Winshell, 1970), and by a group of N-acyl substituted ureido derivatives of ampicillin (the acylureidopenicillins) which include azlocillin, mezlocillin and

Fig. 1.2 The structures of benzylpenicillin and cephalosporin C showing the fused ring systems and side chains



From Greenwood (1989)

piperacillin (Drusano *et al.*, 1984). A 6-α-methoxy derivative of ticarcillin, temocillin, has been developed with properties strikingly different to its parent. Temocillin is highly resistant to a wide range of β-lactamases but virtually inactive against *Pseudomonas aeruginosa* (Jules and Neu, 1982). The major groups of penicillins, with examples, are given in Fig. 1.3.

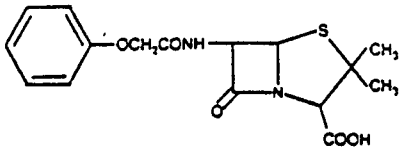
The cephalosporins constitute a large and expanding group of compounds based on cephalosporin C, a fermentation product of a *Cephalosporium* mould (Fig. 1.2), first described by Newton and Abraham (1955). All cephalosporins are derivatives of 7-aminocephalosporanic acid which is obtained from cephalosporin C by chemical means. This molecule can be modified at the C-3 and 7 amino position enabling a large number of derivatives to be produced. In general, alterations at the C-3 position affects pharmacokinetic behaviour and metabolic stability, and changes at the C-7 position affect anti-bacterial activity and β-lactamase stability. The cephalosporins are broad-spectrum compounds, although major differences in their individual spectra of activity exists.

The most commonly used classification of the cephalosporins is the division into three groups or 'generations' based on antibacterial activity and resistance to β-lactamases. 'First generation' compounds were those initially developed and consist of parenteral and oral compounds of moderate antimicrobial activity which are resistant to β-lactamases from staphylococci but are unstable to many of those from enterobacteria. Examples of this group are cephalothin (Griffith and Black, 1964) and cephalexin (Wick, 1967). In the late 1970s, 'second generation' cephalosporins were developed which are a group of parenteral compounds, such as cefuroxime (O'Callaghan *et al.*, 1976) and the cephamycins,

Fig. 1.3 The main groups of penicillins (other than benzylpenicillin) with examples

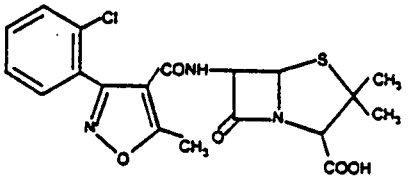
1) Oral compounds resembling benzylpenicillin

Phenoxymethylpenicillin
(penicillin v)



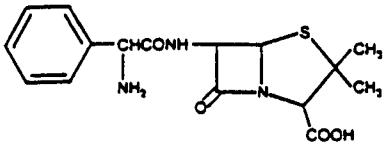
2) Penicillins resistant to staphylococcal β-lactamases

Cloxacillin

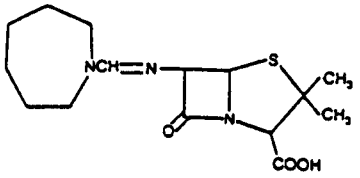


3) Broad spectrum penicillins

Ampicillin

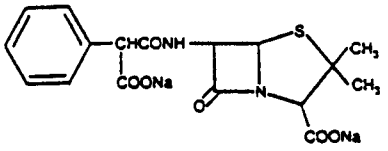


Mecillinam



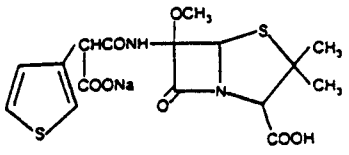
4) Penicillins active against *Pseudomonas aeruginosa*

Carbenicillin



5) β-lactamase resistant penicillins

Temocillin



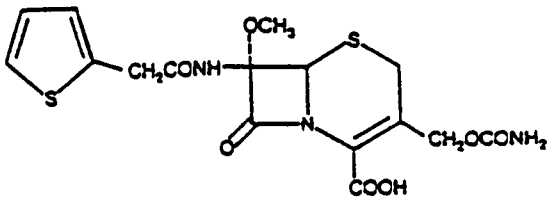
From Lambert and O'Grady (1992)

cefotetan and cefoxitin (Kosmidis *et al.*, 1973) (Fig.1.4); they exhibit moderate antibiotic activity but show greater resistance to a wide range of β-lactamases. More recently, the 'third generation' of parenteral and oral cephalosporins have been developed which combine almost complete stability to most β-lactamases with potent antimicrobial activity. The first to be introduced was cefotaxime (Hamilton-Miller *et al.*, 1978); other examples are ceftazidime, ceftriaxone and ceftizoxime. Latamoxef, a 7-methoxy-oxacephem, is also a member of this group (Otsuka, 1981) (Fig. 1.4). The 'third generation' cephalosporins also include compounds, such as cefoperazone and cefsulodin, which are parenteral antibiotics with moderate to poor activity against enterobacteria. They are active against *P.aeruginosa* and stable to many β-lactamase types (King *et al.*, 1980). The chemical structure of examples from each generation of cephalosporins is shown in Fig. 1.5.

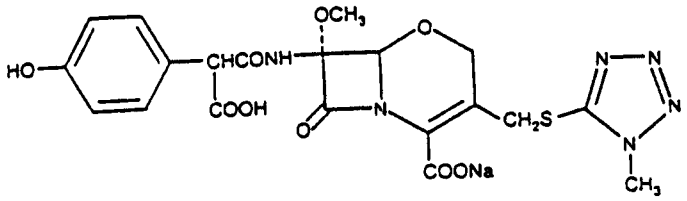
Some semi-synthetic cephalosporins previously mentioned, such as cefoxitin and latamoxef, and certain isoxazolyl penicillins, for example cloxacillin, have the ability to inhibit the activity of a narrow range of β-lactamases. There are other β-lactam antibiotics, with structures fundamentally different from those of penicillins and cephalosporins, which have potent inhibitory activity against a wide range of β-lactamases. These include clavulanic acid, a clavam or oxapenam, and sulbactam, a penicillanic acid sulphone (Fig. 1.6). Both these compounds are irreversible β-lactamase inhibitors, or 'suicide' inactivators, the enzyme and inhibitor initially interacting competitively and then progressively uniting into a complex in which both are inactivated (Bush and Sykes, 1983). Clavulanic acid and sulbactam possess generally poor antibacterial activity but they have

Fig. 1.4 The chemical structure of cefoxitin and latamoxef

Cefoxitin



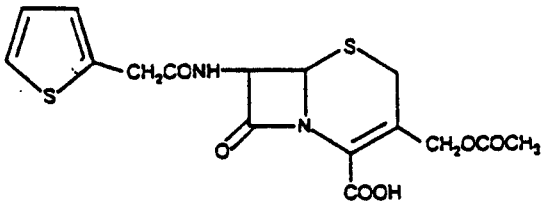
Latamoxef



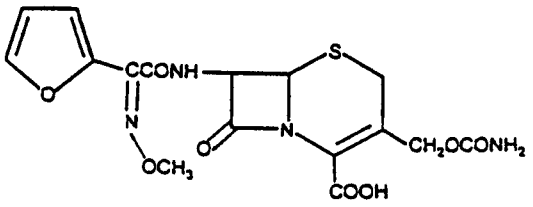
From Lambert and O'Grady (1992)

Fig. 1.5 The chemical structure of examples from the three generations of cephalosporins

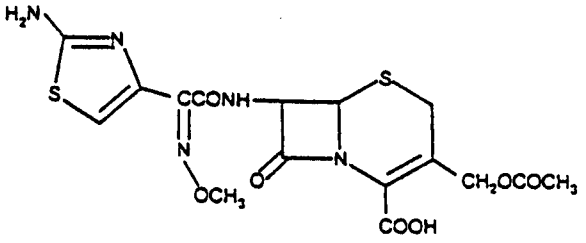
1st Cephalothin



2nd Cefuroxime



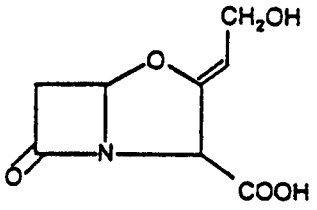
3rd Cefotaxime



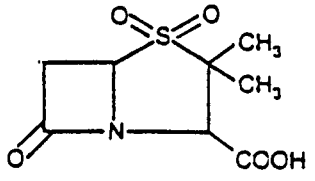
From Lambert and O'Grady (1992)

Fig. 1.6 The chemical structure of clavulanic acid and sulbactam

Clavulanic acid



Sulbactam



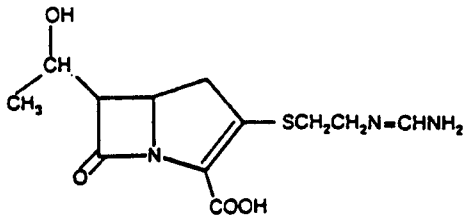
From Lambert and O'Grady (1992)

favourable pharmocokinetic properties enabling them to be used as β-lactamase inhibitors in combination with antimicrobially active, β-lactamase susceptible, β-lactam antibiotics. Clavulanic acid is combined with amoxycillin to give the formulation co-amoxiclav or with ticarcillin. Sulbactam is linked through the carboxyl groups with ampicillin as sultamicillin, and is also available in an unlinked combination with ampicillin (Lambert and O'Grady, 1992). Also, tazobactam, a triazolymethyl penicillanic acid sulphone β-lactamase inhibitor which acts on a variety of clinically important β-lactamases, has been described. It has been shown to markedly enhance the bactericidal activity of piperacillin (Higashitani *et al.*, 1990). Payne *et al.* (1994) recently reported on the comparative activities of clavulanic acid, sulbactam and tazobactam against clinically important β-lactamases. Clavulanic acid and tazobactam were shown to be more active than sulbactam against conventional-spectrum and extended spectrum β-lactamases. There were no differences between the overall activities of tazobactam and clavulanic acid against these enzymes, although differences in their inhibition profiles were observed.

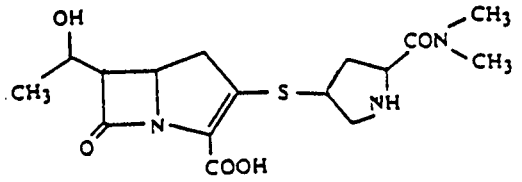
The only carbapenem presently available commercially, imipenem (N-formimidoyl-thienamycin) (Fig. 1.7), is also a β-lactamase inhibitor but more importantly has the additional property of potent activity against a wide range of Gram positive and Gram negative aerobes and anaerobes (Kropp *et al.*, 1985). Although imipenem is stable to most β-lactamases, it is readily hydrolysed by a dehydropeptidase located in the kidney and is therefore administered with a dehydropeptidase inhibitor, cilastatin (Drusano, 1986). Two other carbapenems have been recently developed, meropenem (Fig. 1.7) and biapenem. These have antibacterial spectra similar to that of

Fig. 1.7 The chemical structure of imipenem, meropenem and aztreonam

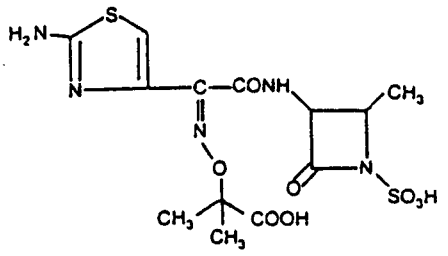
imipenem



meropenem



aztreonam



From Edwards *et al.* (1989) and Lambert and O'Grady (1992)

imipenem but are resistant to hydrolysis by human renal dehydropeptidase (Edwards *et al.*, 1989; Neu *et al.*, 1989; Sader and Jones, 1993; Aldridge *et al*, 1994).

The monobactams, such as aztreonam, are monocyclic β-lactam-1-sulphonic acids having a β-lactam ring but no fused ring system (Fig. 1.7). In contrast to imipenem, aztreonam shows little activity against Gram positive organisms and strict anaerobes, but potent activity against Gram negative bacteria including *P.aeruginosa* (Livermore and Williams, 1981). Aztreonam is not capable of β-lactamase inhibition but is stable to the plasmid-mediated β-lactamases of most Gram negative bacteria (Bush *et al.*, 1982)

The final group of β-lactam agents is the carbacephem class, a notable example of which is loracarbef; a semi-synthetic, orally absorbed antibiotic. This compound has been shown to have broad antibacterial activity with improved β-lactamase stability compared to that of some orally administered cephalosporins (Pelosi and Fontana, 1988).

1:6 In-vitro susceptibility of *Bacteroides* species to β-lactams and other antibiotics

Bacteroides species are almost invariably insusceptible to benzylpenicillin, with minimum inhibitory concentrations (MICs) usually exceeding 16 mg/l (Duerden, 1990). This contrasts with most other clinically important anaerobic bacteria including *Prevotella* spp. and *Porphyromonas* spp. which are normally very susceptible to benzylpenicillin. The other penicillins and the cephalosporins (excluding the related cephamycins and oxa-cephems) also generally show low activity towards *Bacteroides* species (Sutter and Finegold,

1976; Rolfe and Finegold, 1981; Clarke and Zemcov, 1983). However, bacteroides are more susceptible to some of the acylureidopenicillins, such as piperacillin and azlocillin, with typical MICs for *B.fragilis* of 8 mg/l (Cuchural *et al.*, 1990). The rate of resistance to piperacillin in the USA has been reported between 8% and 16%, depending on the geographical source of the isolates (Cuchural *et al.*, 1988a).

Full sensitivity to benzylpenicillin and other β-lactam agents can normally be restored by the addition of β-lactamase inhibitors. Clavulanic acid renders most *Bacteroides* species susceptible. In one study the MIC_{50} (minimum concentration required to inhibit at least 50% of the strains) of amoxycillin against *B.fragilis* strains was reduced from 32 mg/l to 0.25 mg/l, although there was little effect on the susceptibility of *B.distasonis* (Lamothe *et al.*, 1984). Similarly, sulbactam is capable of restoring the activity of β-lactam antibiotics against *Bacteroides* species. For example, the MIC_{50} of ampicillin for clinical isolates was shown by Wexler *et al.* (1985) to be reduced from 64 mg/l to 2 mg/l in the presence of sulbactam. Acar *et al.* (1993) described piperacillin-resistant strains of *B.fragilis* which were susceptible to the piperacillin/tazobactam combination, the MICs of piperacillin being reduced from 128 mg/l to 0.25 mg/l.

The cephamycins, cefoxitin and cefotetan, are more active than conventional cephalosporins against *Bacteroides* species, with MIC_{50} of 8 mg/l (Cuchural *et al.*, 1990). Low-level resistance to cefoxitin (MIC 32 mg/l) was recognised in bacteroides immediately after the introduction of this drug in the late 1970s (Olsson-Liljequist *et al.*, 1980), while high-level resistance (MIC ≥64 mg/l) was first documented in 1983 (Cuchural *et al.*, 1983). Resistance, however, is

uncommon; in a recent European study 3% of *Bacteroides* species were found to be resistant to cefoxitin and 4% of *B.fragilis* strains resistant to cefotetan, at a break point of 32 mg/l (Phillips *et al.*, 1992). *Bacteroides* species are also susceptible to the oxa-cephem, latamoxef, with a greater in-vitro activity compared to cefoxitin as shown by the MIC_{50} of 4 mg/l (Cuchural *et al.*, 1990). The activity of the more recently developed cephamycin, cefminox, against species of *Bacteroides* is comparable to that of latamoxef and superior to that of cefoxitin (Watanabe *et al.*, 1985). In general, *B.fragilis* and *B.vulgatus* are more susceptible to these antibiotics than *B.ovatus*, *B.distasonis* or *B.thetaiotaomicron* (Jenkins *et al.*, 1982; Betriu *et al*, 1990).

Potent activity is displayed by the carbapenems against *Bacteroides* species, with MIC_{50}s of imipenem and meropenem of 0.06 mg/l and 0.12 mg/l respectively (Brown *et al.*, 1981; Martin *et al.*, 1982; Edwards *et al.*, 1989). Imipenem resistance is low, with less than 4% of bacteroides strains reported as insusceptible (Eley and Greenwood, 1986a; Betriu *et al.*, 1992; Phillips *et al.*, 1992; Goldstein *et al.*, 1993). The carbapenems, together with the clavulanate potentiated penicillins, are the most active β-lactam compounds against *Bacteroides* species (Breuil *et al*, 1989; Betriu *et al.*, 1992; Dubreuil *et al.*, 1992). As described in section 1:5, these β-lactam antibiotics also possess a good spectrum of activity against aerobic bacteria.

Of the non-β-lactam antibiotics, the nitroimidazoles have good activity against obligate anaerobic bacteria, with an MIC_{50} of metronidazole for *Bacteroides* species of 0.5 mg/l (Cuchural *et al.*, 1990; Mastrantonio *et al.*, 1994). Resistance of *Bacteroides* species to metronidazole is rarely reported and may sometimes be due to

inadequate anaerobiosis (Phillips *et al.*, 1981; Brogan *et al.*, 1989). Metronidazole, tinidazole and other 5-nitro imidazole compounds share similar activities which do not extend to facultative anaerobes or aerobes (Reynolds *et al.*, 1975).

The lincosomide, clindamycin (and to a lesser extent its parent compound lincomycin) is highly active against *Bacteroides* species (MIC_{50} <0.06 mg/l) and many other anaerobes (Appelbaum and Chatterton, 1978; Cuchural *et al.*, 1990). These antibiotics also possess good activity against staphylococci and streptococci. An increasing problem of clindamycin resistance in *Bacteroides* species has, however, been reported in the U.S.A., Spain and France (Cuchural *et al.*, 1981; Reig *et al.*, 1984; Breuil *et al.*, 1989).

Chloramphenicol and the related thiamphenicol have a broad antibacterial spectrum with good activity against anaerobic bacteria. *B.fragilis* is modestly susceptible to chloramphenicol, with MICs between 1 mg/l and 8 mg/l (Phillips *et al.*, 1992). Resistance in *B.fragilis* is rare, and is due to acetylation of the antibiotic or reduced permeability (Lambert and O'Grady, 1992).

The usefulness of the macrolides, in particular erythromycin, in anaerobic infections is questionable because of the in-vitro resistance, especially among *B.fragilis* strains. Assessment of the clinical potential of macrolides is difficult from in-vitro data because of the effect of variables in the test system, such as pH, on bacterial susceptibility (Greenwood *et al.*,1991).

Aminoglycosides, glycopeptides and quinolones show low or unreliable activity against many bacteroides strains at concentrations achievable therapeutically. The tetracyclines exhibit useful activity, but are associated with high rates of resistance against *Bacteroides* spp.; the MIC of tetracycline for over 60% of clinical isolates was

found to exceed 4 mg/l, in a recent European study (Phillips *et al.*, 1992). These agents are, therefore, not regarded as useful agents for use in infections associated with *Bacteroides* species.

There is variation in sensitivity amongst the species of bacteroides. *B.fragilis* tends to be more susceptible than the other species to β-lactam antibiotics and clindamycin (Jacobus *et al.*, 1989; Phillips *et al.*, 1992). The in-vitro activities of selected antibiotics against *Bacteroides* species are displayed in Table 1.2.

1:7 Penetration of the antibiotic into the bacterial cell

The ability of an antibiotic to reach its site of action is a prerequisite for drug action. In Gram negative bacteria, the outer membrane presents a physical barrier to the penetration of antibiotics into the periplasmic space. Proteins typically make up almost half of the dry weight of an outer membrane and, with the exception of the peptidoglycan-bound and free forms of lipoprotein, are individually named as Omp proteins, after the genes that encoded them. The outer surface of the membrane is largely occupied by lipopolysaccharide, while phospholipid makes up the inner surface (Hancock and Poxton, 1988).

The penetration of β-lactam antibiotics through the outer membrane of *Escherichia coli* has been shown to occur through aqueous channels formed by porin proteins, such as Omp C, Omp F and Pho E (Harder *et al.*, 1981). These proteins are normally arranged as trimers with each monomeric unit, of molecular weight between 28 kDa and 48 kDa, contributing a channel. The three seperate openings of the Omp C and Omp F channels on the outer surface of the outer membrane merge into a single channel near the

Table 1.2 In vitro activity of selected antibiotics against *Bacteroides* species

Antibiotic	Range	MIC (mg/l) MIC_{50}	MIC_{90}
Imipenem	<0.06-8	0.06	1
Amoxycillin-clavulanate	0.5-8	1	2
Ticarcillin-clavulanate	<0.06->128	1	16
Latamoxef	<0.12->128	4	128
Cefoxitin	0.5-128	8	16
Cefotetan	0.5->128	8	128
Ceftizoxime	<0.25->128	8	128
Cefotaxime	0.5->128	32	>128
Cefoperazone	0.5->128	32	>128
Ceftazidime	2->128	>128	>128
Piperacillin	0.5->128	8	>128
Benzylpenicillin	0.5-≥128	16	64
Clindamycin	<0.06->256	<0.06	2
Tetracycline	0.125-64	16	32
Chloramphenicol	0.5-8	4	8
Metronidazole	<0.25-8	0.5	1

From Cuchural *et al.* (1990) and Mastrantonio *et al.* (1994)

mid-point of the membrane in contrast to the non-branching transmembrane channels formed by Pho E protein molecules. The porins allow diffusion across the membrane of small hydrophilic compounds within a limited size range (generally up to 600 daltons) governed by the size of the channel. Most porins do not demonstrate chemical selectivity for different substances, but they can be either cation or anion selective. The cation selective Omp F channel in *E.coli* favours diffusion of zwitterionic over anionic antibiotics, whereas for the anion selective Pho E channel this situation is reversed. The relative proportions of these proteins in the outer membrane, and the proportion of them that form active pores, vary with the growth conditions (Hancock, 1987; Hancock and Poxton, 1988).

The outer membrane protein composition of *Bacteroides* species is more complex than that of *E.coli* (Diedrich and Martin, 1981; Kotarski and Salyers, 1984), and until recently no specific porin molecules in bacteroides had been identified. However, Wexler *et al.* (1992), using various sugars in a liposome assay, reported pore-forming ability of a fraction isolated from the outer membrane of a *B.distasonis* strain which was identified as a heat-modifiable protein.

The rank order of the ability of selected β-lactam antibiotics to permeate the outer membrane of *B.fragilis* was reported by Cuchural *et al.* (1988b) with cephaloridine the most rapid followed by imipenem, cefotaxime, cefoxitin, cephalothin and latamoxef. They also investigated factors influencing permeability and found results similar to those described by Yoshimura and Nikaido (1985) for *E.coli*, in which increases in negative charge and molecular weight are associated with decreased antibiotic uptake. However, unlike the situation in *E.coli*, increased drug hydrophobicity was associated

with increased uptake by *B.fragilis*. It has been proposed that the different effects of hydrophobicity on permeability are due to dissimilarities in the outer membrane lipopolysaccharide composition. The contribution of lipopolysaccharide to the barrier effect of the outer membrane of *E.coli* has been highlighted by Hiruma *et al.* (1984).

1:8 Mode of action of β-lactam antibiotics

The β-lactam antibiotics act by selectively interfering with the cross linking reaction which gives rigidity to the bacterial peptidoglycan within the cell wall. In Gram negative bacilli, bacterial death is secondary to this event as continued growth leads to osmotic rupture of cells which are not protected by an intact wall (Greenwood and O'Grady, 1972; Lambert and O'Grady, 1992).

The mechanism of this inhibition was highlighted by Spratt (1975) who observed that bacterial cell membranes contain several proteins that are able to bind penicillin and other β-lactam antibiotics. These proteins, known as Penicillin Binding Proteins or PBPs, covalently bind β-lactam agents, and are involved in enzymatic activity in peptidoglycan synthesis.

For *E.coli*, the species most extensively studied, seven PBPs have been identified, 1a, 1b, 2, 3, 4, 5 and 6, numbered in descending order of molecular weight. PBPs 1a, 1b, 2 and 3 represent transpeptidase enzymes which are involved in the different forms of cross linkage of glycopeptide polymers essential for growth, septation and division. PBP 1a and 1b are associated with cell elongation, and inhibition of these enzymes results in spheroplast formation and rapid lysis. The shape of the bacterial cell is maintained by PBP 2, selective inhibition of this enzyme causing osmotically stable ovoid and round forms. PBP 3 is required for septum formation

at division and inhibition of this PBP results in the production of filamentous forms. Inhibition of any or all of these essential binding proteins results in cell death preceded by the characteristic morphological change (Spratt, 1977a). The other PBPs 4, 5 and 6 are carboxypeptidases which may control the extent of cross linkage in the cell wall. They appear to be non-essential and unconnected with the antibacterial effect of β-lactams (Amanuma and Strominger, 1980).

The β-lactam antibiotics vary with regard to their relative affinities for the essential binding proteins 1, 2 and 3 of *E.coli* K-12. Few act primarily through PBPs 1a and 1b, although, at low concentrations, cephaloridine binds preferentially to PBP 1a (Curtis *et al.*, 1979). Some β-lactam agents, including cephalexin, cephradine, temocillin and aztreonam, bind only to PBP 3. When present in sufficient concentrations, however, most other β-lactam antibiotics also bind to other sites including PBPs 1a, 1b and 2 (Lambert and O'Grady, 1992). Imipenem and meropenem bind preferentially to PBP 2, but they also bind to PBP 1. Meropenem, unlike imipenem, also has high affinity for PBP 3 (Kitzis *et al.*, 1989). In contrast, mecillinam binds exclusively to PBP 2 (Spratt, 1977b).

The PBPs of *Bacteroides* species have not been studied so extensively as those of *E.coli* and these findings often appeared conflicting and inconclusive. Botta *et al.* (1983) and Georgopapadakou *et al.* (1983) investigated the PBP profile of *B.fragilis* strains and consistently observed three PBPs with molecular weights between 76 kDa and 53 kDa, and 100 kDa and 68 kDa respectively, although additional PBPs of 78 kDa and 32 kDa were occasionally detected by Georgopapadakou *et al.*. Four

species of bacteroides were examined by Piddock and Wise (1986) who found five PBPs in *B.fragilis*, *B.thetaiotaomicron* and *B.ovatus*, and six PBPs in *B.vulgatus*. All species studied possessed three major high molecular weight PBPs, 1, 2 and 3 between 58 kDa and 82 kDa. PBP 1 could be divided into two components, 1a and 1b, for all the species tested except for *B.fragilis* which had a third component 1c. In addition, with the exception of *B.fragilis*, all species examined possessed low molecular weight PBPs below 50 kDa. Yotsuji *et al.* (1988) also reported that *B.fragilis* had five high molecular weight PBPs, between 94 kDa and 72 kDa. More recently Wexler and Halebian (1990) investigated the PBPs of nine *Bacteroides* species and found that each species had a distinctive PBP pattern, although variations within species was seen. Generally, five PBPs were visualised, ranging in molecular weight from 90 kDa to 40 kDa, with one or more bands in the PBP 1 region and PBP 2 appearing fainter than the other PBP bands.

The PBPs of *Bacteroides* species differ from those of *E.coli* in terms of affinity for β-lactam antibiotics and in the morphological consequences of inhibition of these proteins. The primary target in bacteroides for most β-lactam antibiotics is PBP 2 which is involved in septation and corresponds to PBP 3 of *E.coli*. The PBP 1 complex is usually the secondary target site and is associated with cell elongation corresponding to PBP 1 of *E.coli*. Compounds such as imipenem and meropenem bind initially to PBP 3, causing round cells, and then to PBP 2. PBP 3 in bacteroides, therefore, is equivalent to PBP 2 in *E.coli* and is involved in cell shape. Imipenem also binds to PBP 1 at concentrations correlating with the MIC. (Georgopapadakou *et al.*, 1983; Piddock and Wise, 1986).

1:9 Mechanisms of resistance to β-lactam antibiotics

In order to inhibit Gram negative bacteria, β-lactam antibiotics must be able to penetrate the outer membrane, pass through the periplasmic space, and arrive with undiminished potency at the PBPs in the cytoplasmic membrane. Covalent binding with the PBPs has then to occur for the lethal affect to take place. Mechanisms of resistance which interfere with this chain of events are: inactivation of the antibiotic; blockage of transport of the agent into the cell; and alteration of target sites.

1:10 Inactivation of the antibiotic by enzymic attack

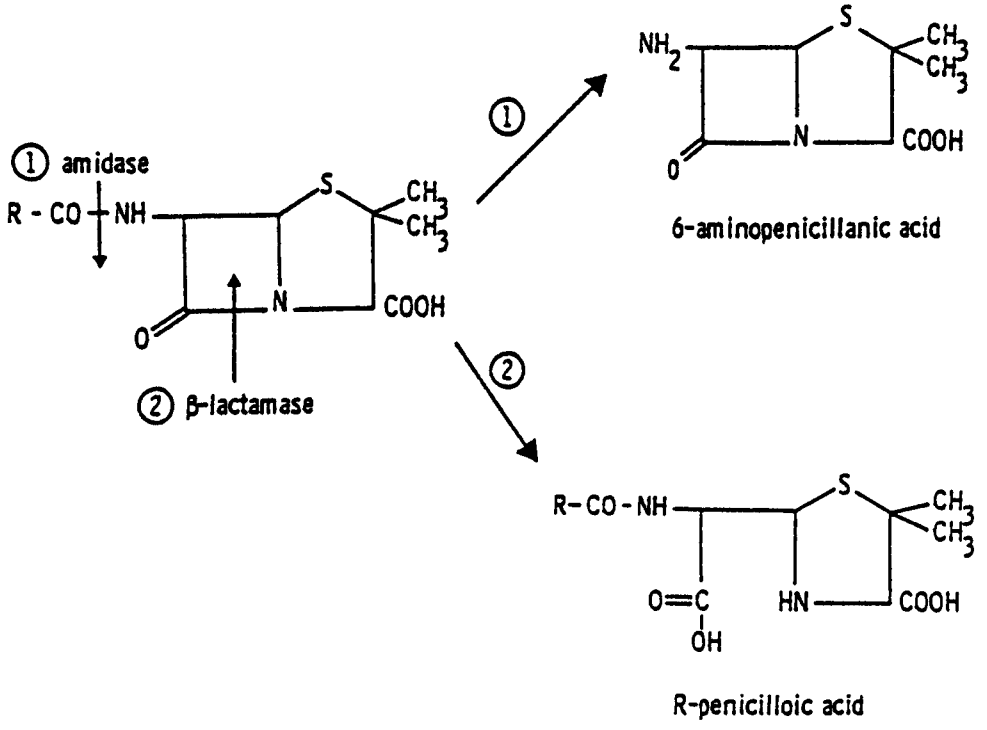
The most common mechanism of resistance to β-lactam antibiotics is degradation of these compounds by the bacterial enzymes amidases, esterases and, most importantly, β-lactamases. The sites of enzymic attack in penicillins and cephalosporins are shown in Figs. 1.8 and 1.9.

Amidases attack penicillins by cleaving the CO-NH bond at the 6-amino position of the β-lactam ring resulting in 6-aminopenicillanic acid which retains some antibacterial activity. The corresponding cephalosporin amidase also exists. These enzymes are not thought to play an important role in β-lactam resistance *in vivo*.

Deacetylation of cephalosporins containing an acetoxymethyl group side chain at the 3 position of the dihydrothiazine ring, for example cefotaxime, can take place as a result of esterase activity in the mammalian liver and kidney. The end products of this reaction exhibit reduced antibacterial activity, as in the case of desacetylcefotaxime. The role of esterases in cephalosporin resistance *in vivo* is not clear.

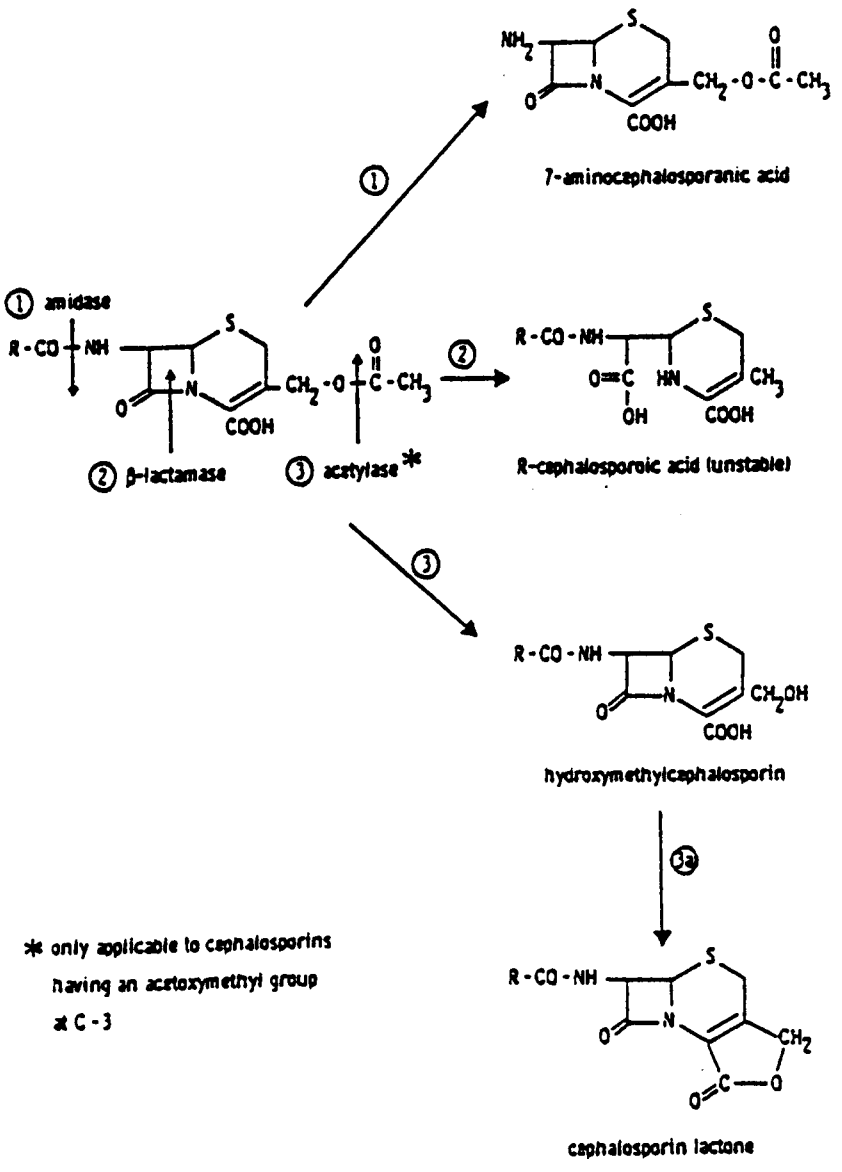
The most important type of resistance to β-lactam antibiotics in

Fig. 1.8 Sites of enzymic attack in penicillins



From Greenwood (1982)

Fig. 1.9 Sites of enzymic attack in cephalosporins



From Greenwood (1982)

clinical medicine is that due to β-lactamase enzymes. These enzymes hydrolyse the β-lactam ring and render the antibiotic inactive. β-Lactamases hydrolyse penicillins to form the corresponding penicilloic acid. Cephalosporins are hydrolysed to produce a cephalosporoic acid which survives only transiently before breakdown into smaller fragments (Sykes and Matthew, 1976).

1:11 Classification of β-lactamases

The β-lactamases of Gram positive and Gram negative bacteria differ fundamentally. Gram positive organisms produce only a few types of inducible exoenzymes which inactivate most penicillins, except for the isoxazolylpenicillins, methicillin and nafcillin, and are much less active against most cephalosporins (Greenwood, 1986).

In contrast, β-lactamases of Gram negative bacteria are a large heterogeneous group with diverse physico-chemical properties which are soluble enzymes largely located in the periplasmic space. Jack and Richmond (1970) presented the first comprehensive classification scheme of β-lactamases from Gram negative organisms. The parameters used for characterising these enzymes were substrate profiles, inhibition by p-chloromercuribenzoate (pCMB) and cloxacillin, the neutralisation by an antiserum prepared against β-lactamase from *E.coli* with a TEM plasmid, and their electrophoretic mobility in starch gel at pH 8. This system was modified by Richmond and Sykes (1973) mainly by adding other β-lactam antibiotics, carbenicillin and cephalexin, to the substrate profiles and by including data on molecular weight. By these criteria, β-lactamases were differentiated into five main classes, I to V. This scheme was then expanded by Sykes and Matthew (1976) by including pI values derived from isoelectric focusing and emphasizing the genetic

location of these enzymes (Table 1.3).

The newer methodologies of amino acid and nucleotide sequencing, together with enzymatic analysis, have been used to distinguish three evolutionary classes of β-lactamases (Ambler, 1980). Class A β-lactamases have a serine residue at their active site, have molecular weights of around 29 kDa, show significant amino acid sequence homology and preferentially hydrolyse penicillins. The common plasmid-encoded TEM-1 and TEM-2 enzymes are included in this group. The class B β-lactamases are metallo-enzymes with molecular weights of about 23 kDa, which require a metal co-factor and attack cephalosporins. Class C enzymes are high molecular weight proteins, approximately 39 kDa, which incorporate the chromosomal mediated cephalosporinases refered to as class I in the Richmond and Sykes classification scheme. They also have serine at their active site, but have no sequence homology with class A β-lactamases.

More recently, Bush (1989a) has put forward an extended classification scheme for β-lactamases. The criteria used for this scheme was based on recommendations made by Bush and Sykes (1986) which include substrate and inhibitor profiles, physical data, inducibility characteristics and genetic location. By these means nine types of β-lactamases are defined, 1, 2a, 2b, 2b',2c, 2d, 2e, 3 and 4 (Table 1.4). Group 1 are cephalosporinases which are not inhibited by clavulanic acid and are almost always chromosomal in origin. Their molecular weights are generally over 30 kDa and their isoelectric points are basic. Group 2a are penicillinases inhibited by clavulanic acid which include those from Gram positive organisms. β-Lactamases from group 2b exhibit broad spectrum activity,

Table 1.3. Summary of the classification of β-lactamases from aerobic Gram negative bacteria

Class	Substrate characteristics	Genetic location	Genetic control	Inhibited by cloxacillin	Inhibited by pcmb*	Found in
I	cephalosporins	chromosome	inducible/ constitutive	+	- * *	*E.coli* *Proteus* spp. *Enterobacter* spp. *Citrobacter* spp. *Pseudomonas* spp. *Serratia* spp.
II	penicillins	chromosome	constitutive	+	-	*E.coli* *P.mirabilis*
III#	penicillins/ cephalosporins	plasmid	constitutive	+	-	many *Enterobacteriaceae* *Pseudomonas* spp. *H.influenzae* *N.gonorrhoeae*
IV	penicillins/ cephalosporins	chromosome	constitutive	-	+	*Klebsiella* spp. *Enterobacter* spp.
V	penicillins including methicillin and isoxazolylpenicillins	plasmid	constitutive	-	-	*Pseudomonas* spp.

* pcmb = p-chloromercuribenzoate

** = not inhibited by clavulanic acid

= group III includes the TEM enzymes

Based on Richmond and Sykes (1973)

Table 1.4 General classification scheme for bacterial β-lactamases

Group	Subtitle	Prefered substrate	inhibited by CA[a]	inhibited by EDTA	Representative enzyme(s)
1	CEP-N	Cephalosporins	No	No	Chromosomal enzymes from Gram-negative bacteria
2a	PEN-Y	Penicillins	Yes	No	Gram-positive penicillinases
2b	BDS-Y	Cephalosporins, penicillins	Yes	No	TEM-1, TEM-2, SHV-1
2b'	EBS-Y	Cephalosporins, penicillins, cefotaxime	Yes	No	TEM-3, TEM-5, SHV-2, SHV-3
2c	CAR-Y	Penicillins, carbenicillin	Yes	No	PSE-1, PSE-3, PSE-4
2d	CLX-Y	Penicillins, cloxacillin	Yes[b]	No	OXA-1, PSE-2
2e	CEP-Y	Cephalosporins	Yes	No	*Proteus vulgaris*
3	MET-N	Variable	No	Yes	*Bacillus cereus* II, *Pseudomonas maltophilia* L1
4	PEN-N	Penicillins	No	?[c]	*Pseudomonas cepacia*

a 10 μM clavulanic acid

b Inhibition by clavulanic acid may occur at higher concentrations for some members of the group

c Variable

From Bush (1989a)

hydrolysing both penicillins and cephalosporins, but are inhibited by clavulanic acid. They tend to have molecular weights lower than 30 kDa and include TEM-1 and TEM-2 enzymes. Group 2b' includes β-lactamases capable of hydrolysing the extended-broad spectrum β-lactam antibiotics such as cefotaxime and aztreonam, and are inhibited by clavulanic acid (Bush, 1989b). Groups 2c and 2d are β-lactamases which hydrolyse carbenicillin or cloxacillin respectively and are generally inhibited by clavulanic acid. Group 2e are cephalosporinases separate from group 1 which are inhibited by low concentrations of clavulanic acid. The metallo-enzymes are represented by group 3, which are inhibited by EDTA with activity restored by divalent cations (commonly Zn^{2+}), and are not inhibited by clavulanic acid. Finally, group 4 contains unusual penicillinases which are not inhibited by clavulanic acid (Bush, 1989c).

1:12 β-Lactamases of *Bacteroides* species

Garrod (1955) was the first to report the penicillin-destroying activity of *B.fragilis*, showing that two out of 31 strains examined were able to destroy penicillin in solution. In 1968, Pinkus *et al.* documented the production of β-lactamases by 14 penicillin-resistant strains in a collection of 29 isolates of *B.fragilis*. The first characterisation of a β-lactamase produced by a strain of *B.fragilis* exhibiting high level ampicillin resistance was provided by Anderson and Sykes (1973). They showed that the enzyme was a cephalosporinase rather than a penicillinase, that it was not inducible and that its production correlated with high level resistance to β-lactam antibiotics. Olsson *et al.* (1977) reported that most bacteroides strains (87%) produced small amounts of a constitutive β-lactamase, and 6% of strains produced elevated amounts of β-

lactamase which correlated with high resistance to β-lactam antibiotics. In 1988, Aldridge *et al.* identified 97% of 246 *B.fragilis* isolates from the USA that produced β-lactamases of some type. A more recent survey in the USA has shown that at least 90% of all *B.fragilis* group strains produce β-lactamase and that 25% produce high levels (Cornick *et al.*, 1990). The increasing frequency of isolation of high-level β-lactamase-producing strains suggest a trend towards high-level β-lactamase production in *Bacteroides* species.

The production of β-lactamases by *Bacteroides* species has been shown in some strains to be highly dependent on growth phase: enzyme production increases sharply during the logarithmic phase of growth to reach a maximum in the pre-stationary phase before declining again during the stationary phase (Olsson *et al.*, 1976; Edwards and Greenwood, 1990). Results from studies of the location of β-lactamase in *B.fragilis* indicate that the enzymes are largely cell associated, within the periplasmic space, and can be released by osmotic shock (Britz and Wilkinson, 1978).

Futher characterisation of the common β-lactamases from *B.fragilis* have shown them to have a molecular weight of 30 kDa to 40 kDa, and to be inhibited by cloxacillin, pCMB and clavulanic acid (Olsson *et al.*, 1976; Wise, 1977; Britz and Wilkinson, 1978). Cefoxitin, latamoxef and imipenem are typically resistant to hydrolysis by these enzymes (Brown *et al.*, 1981). Timewell *et al.* (1981) reported on the β-lactamases from *B.fragilis*, *B.thetaiotaomicron*, *B.vulgatus* and *B.uniformis* and found species differences in terms of substrate profile and pI values which ranged between 4.9 for *B.fragilis* and 4.25 for *B.thetaiotaomicron*. They did, however, share the general characteristics previously described.

These properties differ from those of β-lactamases from aerobic

bacteria. Bacteroides β-lactamases do, however, bear some resemblance to the enzymes of Richmond and Sykes class I, except in terms of inhibition by pCMB and clavulanic acid (Timewell *et al.*, 1981). An additional class (VI) of the Richmond and Sykes scheme was put forward by Neu (1986) to accommodate β-lactamases from *Bacteroides* species. Typical β-lactamases produced by *B.fragilis* strains have been placed in group 2e of the Bush classification scheme (Britz and Wilkinson, 1978; Yotsuji *et al.*, 1983; Bush, 1989c). Recently, Rogers *et al.* (1993) characterised the cephalosporinase gene *cepA* from *B.fragilis* with high specific activity and showed that these typical β-lactamases formed a distinct subgroup of Ambler class A enzymes.

A wide variety of β-lactamases from bacteroides which are not typical of those commonly encountered have been reported. Also, although most of the genes for β-lactamases in the bacteroides are located on the chromosome, plasmid mediated enzymes have been identified. Sato *et al.* (1982) described a β-lactamase produced by a *B.fragilis* strain which was a potent penicillinase, had weak cephalosporinase activity, was inhibited by pCMB and had an isoelectric point of 6.9. In this case, β-lactamase production was found to be transferable and this was considered to be plasmid mediated. This enzyme has been designated a member of group 2d of the Bush classification scheme (Bush, 1989c). Cefoxitin resistance transfer has been reported in a strain of *B.thetaiotaomicron*, although the mechanism of the resistance for this isolate was not determined (Rashtchian *et al.*, 1982). Cuchural *et al.* (1983) also described atypical β-lactamases from strains of *B.fragilis* which were able to inactivate cefoxitin and were resistant to clavulanic acid. One of these

strains, TAL 4170, was later shown to transfer β-lactamase-mediated cefoxitin resistance to a susceptible *B.fragilis* recipient by conjugation (Cuchural *et al.*, 1986a). Another cephalosporin-hydrolysing *B.fragilis* β-lactamase which was not inhibited by clavulanic acid has been reported and assigned to group 1 of the Bush classification scheme (Sato *et al.*, 1980; Bush, 1989b).

A 'new class' of β-lactamase from *B.fragilis*, which inactivated a wide range of β-lactam substrates usually considered stable to hydrolysis including cephamycins and carbapenems, was reported by Cuchural *et al.* (1986b). These enzymes were inhibited by the ion chelator ethylenediaminetetraacetic acid (EDTA), and zinc ions completely reversed this inhibition. Clavulanic acid did not inhibit β-lactamase activity. A metallo-β-lactamase with similar properties from a *B.fragilis* strain was reported by Bandoh *et al.* (1991). This enzyme also hydrolysed carbapenems and cephamycins and was not susceptible to clavulanic acid. Inactivation of the enzyme by EDTA was completely reversed by the addition of zinc ions. A β-lactamase described by Ajiki *et al.* (1991) required zinc as a co-factor and hydrolysed imipenem and broad-spectrum cephem antibiotics of the cefotaxime type. These compounds were more rapidly hydrolysed than penicillin G. The most stable β-lactam antibiotics tested were cephamycins. It was concluded that this metallo-β-lactamase belonged to the same category of enzymes reported by Cuchural *et al.* (1986b). More recently, Hedberg *et al.* (1992) also characterised an imipenem hydrolysing metallo-β-lactamase from a *B.fragilis* strain. The substrate profile of this enzyme differed from that described by Bandoh *et al.* (1991), while other characteristics such as inhibition profiles and physical properties were similar. All these enzymes caused substantial resistance to imipenem (MICs ≥100

mg/l), had pI values in the range from 4.5 to 5.2 and molecular weights between 25 kDa and 33 kDa when determined by sodium dodecylsulphate polyacrylamide gel electrophoresis. These metallo-β-lactamases belong to Ambler's molecular class B and the functional Bush group 3 (Yang *et al.*, 1992). Earlier, Yotsuji *et al.* (1983) reported a potent β-lactamase produced by a *B.fragilis* strain which had a similar substrate profile to that described by Cuchural *et al.* (1986b), being capable of hydrolysis of cephamycin derivatives and imipenem, and not susceptible to clavulanic acid. However, EDTA inhibition was not mentioned in their report. Similarly, Eley and Greenwood (1986a,b) described three clinical isolates of *B.fragilis* which produced β-lactamases capable of rapid hydrolysis of imipenem and were resistant to clavulanic acid. The affect of EDTA on the activity of these enzymes was not examined. The carbapenemase producing isolates of *B.fragilis* examined by Yotsuji *et al.* (1983) and Eley and Greenwood (1986a,b) showed low to intermediate levels of resistance to imipenem (MICs 0.25 mg/l to 32 mg/l).

Direct sequencing or probing has confirmed the presence of highly homologous metallo-β-lactamase *ccr*A and *cfi*A genes in 11 clinical isolates of *B.fragilis* from USA, UK and France (Rasmussen *et al.*, 1990; Thompson and Malamy, 1990; Rasmussen *et al.*, 1991; Podglajen *et al.*, 1992a). By DNA sequencing analysis, Rasmussen *et al.* (1992) revealed that metallo-β-lactamases produced by three *B.fragilis* strains with different MICs (16-fold range for imipenem) differed at five amino-acid residues. These three enzymes were overexpressed, purified and their kinetic values for a variety of β-lactam antibiotics determined. By these means, it was found that the five amino-acid substitutions only modestly affected the hydrolysing activity of these β-lactamases. Podglajen *et al.* (1990) described the

selection of imipenem resistance, associated with zinc β-lactamases, in *B.fragilis* isolates which initially appeared moderately susceptible to imipenem. By use of a DNA probe they have subsequently shown that 2.2% of the 550 *B.fragilis* clinical isolates studied carried the zinc carbapenemase gene (*cfi*A). Two-thirds of these *cfiA* genes were described as silent, being associated with non highly resistant strains for which the MICs of imipenem were ≤2 mg/l (Podglajen *et al.*, 1992b). It was concluded that differences in susceptibilities were governed by the level of expression of these metallo-β-lactamase genes.

Until recently, transfer of metallo-β-lactamase of bacteroides had not been observed. Genes coding for *B.fragilis* metallo-β-lactamases have been shown by Thompson and Malamy (1990) to be present on the chromosome. However, in 1992, Bandoh *et al.* reported on a strain of *B.fragilis* containing a metallo-β-lactamase gene on a small plasmid tranferable by conjugation. This situation greatly increases the potential for spread of the enzyme.

Imipenem resistance (MIC 16 mg/l) in a *B.distasonis* isolate was attributed by Hurlburt *et al.* (1990) to the production of an unusual imipenem inactivating serine-β-lactamase together with impermeability. This enzyme, with a molecular mass of 160 kDa, was inhibited by clavulanic acid and sulbactam, but not EDTA.

Further attempts have been made to classify the wide variety of β-lactamases encountered in bacteroides strains. Eley and Greenwood (1986b) examined the characteristics of type culture strains of *Bacteroides* species and bacteroides strains known to hydrolyse cefoxitin, latamoxef or imipenem. They defined three broad groups of β-lactamases based on antibiotic degradation and inhibitor

profiles: those enzymes that do not inactivate cefoxitin, latamoxef and imipenem and were susceptible β-lactamase inhibitors; those that hydrolyse cefoxitin and latamoxef but not imipenem and were less susceptible to inhibitors; and those that inactivated all three antibiotics and were not inhibited by β-lactamase inhibitors. In another study, Eley and Greenwood (1986a) characterised the β-lactamases produced in raised amounts by clinical isolates of bacteroides. These enzymes were divided into three groups using the additional criterion of specific cephalosporinase activity (μ moles nitrocefin degraded per min per mg protein): those of low specific activity which were susceptible to β-lactamase inhibitors; enzymes of intermediate specific activity which hydolysed cefoxitin, latamoxef and sometimes imipenem and were insusceptible to inhibitors; and those of high specific activity which were susceptible to β-lactamase inhibitors but incapable of inactivation of β-lactamase stable β-lactam antibiotics.

1:13 Reduction of penetration the antibiotic

The role of porins in the outer membrane permeation of β-lactam antibiotics has been extensively studied in Gram negative aerobic bacteria (Nikaido, 1989). Sawai *et al.* (1982) found diminished uptake of cephazolin in porin-deficient mutant strains of *Proteus mirabilis* and *Enterobacter cloacae*. Mitsuyama *et al.* (1987) examined porins from cefoxitin-resistant mutants of *Proteus*, *Morganella* and *Providencia* species and established their role in the permeation of β-lactam antibiotics. Similarly, Godfrey and Bryan (1987) found diminished permeation of β-lactam antibiotics in a mutant of *P.aeruginosa* with a structurally modified porin.

The role of permeability barriers in β-lactam antibiotic resistance in *Bacteroides* species has been observed indirectly.

Results from crypticity measurements (the ratio of β-lactamase activity of broken cells to the activity of intact cells), with cephaloridine as substrate, have indicated that limited outer membrane permeability to β-lactam antibiotics contributed to resistance in certain strains of *B.fragilis* (Olsson *et al.*, 1979). Dornbusch *et al.* (1980) examined strains of *B.fragilis* with decreased sensitivity to cefoxitin and found no correlation between β-lactamase production and resistance to cephamycins. They postulated that changes in the cell wall, causing decreased penetration of the antibiotic, could be a resistance factor. Crosby and Gump (1982) used EDTA to increase the permeability of the cell wall in an attempt to assess the importance of impermeabilty of this wall in terms of antibiotic resistance. The addition of EDTA significantly enhanced the activity of cefoperazone against β-lactamase negative as opposed to β-lactamase positive bacteroides. From this they concluded that cell impermeability was a major mechanism of resistance of cefoperazone in β-lactamase negative isolates. Malouin and Lamothe (1987) found a significant increase of susceptibility of bacteroides to cephalosporins with EDTA, indicating a role for a permeability barrier. Also, Cuchural *et al.* (1986b) reported β-lactamase mediated imipenem resistance in *B.fragilis* associated with a barrier to drug permeation determined by crypticity measurements. Imipenem resistance in *P.aeruginosa* has been shown to be associated with outer membrane protein composition and decreased permeability (Buscher *et al.*, 1987; Trias *et al.*, 1989).

Evidence of the association between cefoxitin resistance and alteration of the porin proteins in *Bacteroides* species was put forward by Piddock and Wise (1987). They described two cefoxitin resistant strains, one of which was *B.fragilis* and the other

B.thetaiotaomicron, in which resistance was not β-lactamase mediated but related to alteration in outer membrane protein profiles together with PBP changes. An outer membrane protein, possibly a porin protein, of molecular weight approximately 50 kDa was absent from both strains.

A correlation between lipopolysaccharide structure and resistance due to lack of permeability of β-lactam antibiotics in *Bacteroides* spp. has not been demonstrated. However, this has been shown with *P. aeruginosa* (Godfrey *et al.*, 1984).

1:14 Alteration of penicillin binding proteins

In many species of aerobic Gram negative bacteria, the modification of PBP affinity has been shown to result in β-lactam antibiotic resistance (Malouin and Bryan, 1986). However, there is less evidence of changes in particular penicillin binding proteins as a resistance mechanism in *Bacteroides* species. *Bacteroides* species are naturally resistant to monobactam antibiotics because of the poor affinity of these compounds for any of the PBPs. Aztreonam has been shown to bind poorly or undetectably to all PBPs of *B.fragilis* (Georgopapadakou *et al.*, 1982).

Georgopapadakou *et al.* (1983) were the first to report reduced affinity for piperacillin, cefoperazone, cefotaxime, ceftazidime and imipenem to PBP 2 in a resistant strain of *B.fragilis*. Changes in the affinity of PBP 1 or PBP 2 in laboratory derived mutants have also been correlated with a decrease in susceptibility to cefoxitin (Piddock and Wise, 1987). Yotsuji *et al.* (1988) proposed that decreased affinity for PBP 3, and not β-lactamase hydrolysis or membrane permeation, was the important factor in the resistance to ceftezole,

cefazolin and cephalothin in a *B.fragilis* strain. More recently, Wexler and Halebian (1990) reported changes in both the PBP-1 complex and the affinity of one of the PBP-1 proteins for cefoxitin between cefoxitin sensitive and resistant strains of *B.thetaiotaomicron*. Resistant strains of *B.uniformis* also showed changes in the PBP-1 complex in comparison with sensitive strains. In addition, they showed that a laboratory derived cefoxitin resistant mutant of *B.distasonis* displayed reduced binding to the PBP-1 complex compared with its wild type parent and cefoxitin sensitive revertant. No obvious changes in outer membrane profiles were detected to indicate changes in permeability.

1:15 Aims of study

This study set out to investigate the mechanisms by which *Bacteroides* species can resist the antibacterial activity of β-lactam antibiotics. It was proposed to determine initially the prevalence of β-lactam resistance by evaluation of current susceptibility patterns of randomly selected clinical isolates of *Bacteroides* species to certain β-lactam antibiotics. The degree of β-lactamase production of these strains was also examined. The findings were compared with those of previous studies to determine any changes in these values with time.

The role of β-lactamases activity in the resistance of the bacteroides isolates was assessed. β-Lactamases from the bacteroides isolates were examined in terms of a range of physical and chemical properties. These findings were used to categorise the β-lactamases in order to clarify the problematic classification of β-lactamases from *Bacteroides* species.

Mechanisms of resistance of bacteroides to β-lactam antibiotics, other than that due to β-lactamase activity, are poorly

defined. The outer membrane structure has only been examined in a few resistant strains. Scanty reports refer to changes in porin proteins in association with antibiotic resistance, and the lipopolysaccharide composition has not been implicated. The PBPs of *Bacteroides* species have not been studied extensively and results have often been conflicting. A few studies have described reduced affinity for β-lactam antibiotics to the PBPs of resistant bacteroides strains. However, only a limited number of antibiotics and *Bacteroides* strains have been investigated.

In an attempt to clarify this situation, mechanisms of resistance were explored in detail in *B.fragilis* strains with increased resistance to carbapenems. Carbapenemase activity was determined and these β-lactamases investigated further. Isolates exhibiting non-β-lactamase mediated resistance were identified and their resistance mechanisms examined. Reduction in antibiotic penetration into the bacterial cell was investigated. The PBPs of these isolates were detected and compared to those of normal sensitive *B.fragilis* strains. The interaction of these means of resistance was also considered.

CHAPTER 2

MATERIALS AND METHODS

2:16 Effects of clavulanic acid and zinc acetate on broth dilution MICs of imipenem

2:17 β-Lactamase crypticity measurements

2:18 Outer membrane protein analysis

2:19 Lipopolysaccharide analysis

2:20 Analysis of penicillin binding proteins

(1) Scintillation counts of gel segments

(2) Fluorography

CHAPTER 2

MATERIALS AND METHODS

2:1 Growth media and incubation conditions

The following media were used throughout the study:

(i) Horse blood agar (blood agar base No. 2 (Unipath Ltd., Basingstoke, Hampshire) containing 7% horse blood (Tissue Culture Services Ltd., Buckingham)).

(ii) Brain Heart Infusion with supplements (BHIS) comprising Brain Heart Infusion broth (Unipath), yeast extract 5 g/l (Unipath), haemin (Sigma Chemical Co. Ltd., Poole, Dorset) 5 mg/l and menadione (Sigma) 1 mg/l. BHIS agar was obtained by the addition of 1% agar No. 1 (Unipath).

Throughout the study, anaerobic incubation was carried out at 37°C in a Mark 2 Don Whitley anaerobic cabinet (Don Whitley Scientific Ltd, Shipley, Yorkshire) with a gas mixure of 10% hydrogen, 10% carbon dioxide and 80% nitrogen.

All media were pre-reduced overnight in the anaerobic cabinet before use.

2:2 Bacterial strains

Clinically significant isolates of anaerobic Gram negative bacilli were collected randomly from specimens submitted to Nottingham Public Health Laboratory in 1989 and 1990. These strains were originally isolated on horse blood agar containing neomycin at a concentration of 60 mg/l. They were then subcultured to horse blood agar with a metronidazole disc (5µg/disc) and checked for purity after

anaerobic incubation for 48 hours.

Bacteroides strains were distinguished from other anaerobic Gram negative bacilli by their ability to hydrolyse aesculin and by their tolerance to bile. They were identified to species level, if possible, by the use of carbohydrate fermentation and indole production tests. These results were compared with the identification scheme described by Duerden (1990) which is summarised in Table 2.1.

Aesculin hydrolysis was tested by the method of Phillips (1976) modified to include a test for bile tolerance. Horse blood agar plates containing 20% bile were spread with a ferric citrate-aesculin solution to give a final concentration of 0.1% aesculin and spot-inoculated. After 48 hours of anaerobic incubation, bile tolerance was detected by growth of the organisms and aesculin hydrolysis by blackening of the medium.

The fermentation of glucose, rhamnose, trehalose, salicin and mannitol was assessed by the methods described by Phillips (1976). A sterile 20% solution of carbohydrate substrate was spread over the surface of a horse blood agar plate and allowed to dry. Test cultures were spot inoculated and the plates incubated anaerobically for 48 hours. A control horse blood plate was also included. Fermentation of the carbohydrate was indicated by a zone of haemolysis.

Equivocal carbohydrate fermentation reactions were repeated with Difco (Bacto) Differentiation Disks (Difco Laboratories, West Molesey, Surrey). The surface of a horse blood agar plate was spread with the test strain suspension in BHIS broth to produce a confluent growth. The filter paper Differentiation Disks, impregnated with a carbohydrate, were placed on the agar (2 per plate) and the plates incubated anaerobically for 48 hours. Carbohydrate fermentation was detected by enhanced bacterial growth around the disc together with

Table 2.1 Identification scheme for *Bacteroides* species

	Production of acid from:					Indole production
Species	Glucose	Rhamnose	Trehalose	Salicin	Mannitol	
B.fragilis	+	-	-	-	-	-
B.ovatus	+	+	+	+	+	+
B.thetaiotaomicron	+	+	+	+	-	+
B.distasonis	+	+/-	+	+	-	-
B.vulgatus	+	+	-	-	-	-
B.uniformis	+	-	+	-	-	+
B.caccae	+	+	+	-	-	-
B.eggerthii	+	+	-	+	-	+
B.merdae	+	-	+	+	-	-
B.stercoris	+	+	-	-	-	+

From Duerden (1990)

a decrease in pH indicated by a colour change to yellow after the addition of one drop of 0.5% phenol red onto the disk.

The test for indole production was carried out as described by Summanen *et al.* (1993). One ml of xylene was added to 2 ml of cooked meat broth (Unipath) culture and mixed well. Ehrlich's reagent was then added and the development of a pink colour indicated a positive reaction.

When the results of the above tests proved difficult to interpret or were persistently equivocal, identification of the bacteria was obtained by the ATB 32 A system (API-bioMerieux, Basingstoke, Hampshire) which consists of 29 cupules containing dehydrated test substrates. The system was used according to the manufacturer's instructions.

Those clinical isolates identified as species of *Bacteroides* were then maintained in duplicate. Pure bacteroides cultures were harvested from horse blood agar plates and a heavy inoculum was emulsified into 1.5 ml of skimmed milk (Unipath) in plastic vials which were then frozen at -70°C.

In addition to these recently isolated clinical strains of bacteroides, five *B.fragilis* strains from previous studies were used in certain sections of the present study (Eley and Greenwood, 1986a,b): *B.fragilis* 0423, originally supplied by V. L. Sutter (Wadsworth Veterans Administration Medical Centre, Los Angeles, USA), *B.fragilis* 2013E, donated by I.N. Simpson (Glaxo Ltd, Greenford, Middlesex) and *B.fragilis* 57, 97 and 119, clinical isolates from the Nottingham Public Health Laboratory. Also, *B.fragilis* NCTC 9344 was used as a control strain throughout the study. These four strains were maintained in skimmed milk at -70°C as previously described.

Test strains were reconstituted by touching the surface of the

frozen skimmed milk bacterial suspension with a hot loop and spreading the bacteria on a horse blood agar plate which was then incubated anaerobically. The vial containing the frozen bacteria was immediately replaced at -70°C after use without thawing.

2:3 Semi-quantification of β-lactamases in intact bacteria

Cephalosporinases were detected by a colour change resulting from the hydrolysis of the chromogenic cephalosporin nitrocefin as described by Aldridge *et al.* (1983). A solution containing nitrocefin 250 mg/l was prepared by dissolving lyophilised nitrocefin (Unipath) in rehydration fluid (Unipath) containing 0.1M phosphate buffer (pH 7.0) and dimethylsulphoxide. Three drops of this solution were added to a microtitration tray well and seeded with several colonies from an overnight culture grown on BHIS agar. The presence of cephalosporinase was indicated by a colour change from yellow to red at room temperature which was quantified visually over a one hour period.

The method of Escamilla (1976), which relies on the pH change resulting from the hydrolysis of benzylpenicillin to penicilloic acid, was used to detect the presence of penicillinase. A penicillin - phenol red reagent was prepared by dissolving 560 mg benzylpenicillin in 4.5 ml of distilled water and adding 0.5 ml of a 0.5% aqueous solution of phenol red. One drop of 1M sodium hydroxide was then added to give a violet colour at pH 8.5. Three drops of the penicillin - phenol red reagent were added to each well of a microtitre tray and seeded as before. The extent of a colour change from red to yellow was visually assessed during incubation at room temperature for one hour.

2:4 Antibiotics and antibiotic titrations

Solutions of benzylpenicillin sodium salt (Glaxo), clavulanic acid lithium salt (SmithKline Beecham Pharmaceuticals, Brentford, Essex), cefoxitin sodium salt (Merck, Sharp and Dohme Ltd, Hoddesdon, Hertfordshire), latamoxef disodium salt (Eli Lilly and Co. Ltd, Basingstoke, Hampshire), sulbactam sodium salt (Pfizer Ltd, Sandwich, Kent) and cloxacillin sodium salt (SmithKline Beecham) were freshly prepared as required in sterile distilled water. Appropriate concentrations of imipenem (Merck, Sharp and Dohme) were dissolved in 0.01 M phosphate buffer, pH 7.0.

Mininum inhibitory concentrations (MICs) were determined for the clinical isolates of bacteroides by the agar incorporation method. Serial doubling dilutions of antibiotic were incorporated in BHIS agar. Overnight cultures of test and control strains in BHIS broth were diluted 1 in 10 with fresh BHIS broth. These were spot inoculated on to the antibiotic-containing plates by use of a multipoint inoculator (Denley Instruments, Billingshurst, Sussex) resulting in an inoculum of approximately 10^5-10^6 bacteria per spot. An antibiotic-free control plate was also included. The plates were incubated anaerobically for 48 hours and the MIC was taken as the lowest antibiotic concentration that completely inhibited growth.

2:5 Preparation of crude β-lactamase extracts

Bacterial growth of each test strain was harvested after 48 hours of anaerobic incubation from two horse blood agar plate cultures with a sterile cotton wool swab. The bacteria were suspended in a glass tube containing 4 ml phosphate buffer (0.02 M; pH 7.0) which was kept cooled in a beaker of iced water. The

bacterial suspensions were then subjected to six bursts of sonication, each of 30 seconds, with cooling periods of 30 seconds between bursts, in a Soniprep 150 ultrasonicator (MSE Scientific Instruments, Crawley, Sussex). The disrupted cells were centrifuged at 4000 rpm for 30 minutes at 4°C in a 'Coolspin' centifuge (MSE). The supernate (crude β-lactamase extract) was divided into 0.5 ml portions and stored at -70°C, or split into 2 ml portions and freeze dried (Freeze Drier 3.5, Birchover Instruments Ltd., Letchworth, Hertfordshire), to be reconstituted later in a concentrated form with phosphate buffer.

2:6 Determination of specific activity

(1) with nitrocefin as substrate

The method used was based on that described by O'Callaghan *et al.* (1972): 1.9 ml of a nitrocefin solution (52 mg/l nitrocefin in 0.02 M phosphate buffer pH 7) and 0.1 ml crude enzyme extract were mixed in a cuvette to give a final nitrocefin content of 0.2 μ moles. The rate of hydrolysis of nitrocefin was measured over a three minute period at 37°C and 482 nm in a spectrophotometer with heated cuvette compartment (UV-160A, Shimadzu, Kyoto, Japan). The enzyme concentration was adjusted to give complete hydrolysis of the substrate in approximately seven minutes. This was achieved by appropriate dilution of the extracts in phosphate buffer when the hydrolysis was too rapid, or with reconstituted freeze dried concentrates when the enzyme activity was too slow .

The total soluble protein content of the crude extract was measured with a Sigma Protein Assay Kit (Sigma) in accordance with the manufacturer's instructions. The procedure is based on a modification of the Lowry protein determination method as described by Peterson (1977).

Specific activity (SA) was calculated from the formula:

$$SA = \frac{\Delta OD/min}{\text{total } \Delta \text{ OD}} \times \frac{\mu \text{ moles nitrocefin present}}{\text{protein content (mg/ml)}} \times \text{enzyme dilution}$$

where ΔOD is the change in optical density. The SA was expressed as: the amount (μ moles) of nitrocefin hydrolysed/min/mg of protein.

(2) with imipenem as substrate

The specific imipenemase activities, defined as μ moles imipenem hydrolysed/min/mg of protein, were assessed for those strains shown to be capable of imipenem hydrolysis. A mixture of 100 μl of concentrated crude cell extract, 200 μl imipenem 250 mg/l (0.16 μmoles) and 700 μl phosphate buffer was placed in a 1 ml cuvette and maintained at 37°C in a spectrophotometer (UV-160A, Shimadzu). The rate of change in absorbance at 299 nm was measured over a five minute period and the degree of concentration of crude cell extract chosen gave complete hydrolysis of imipenem within one hour. The change in absorbance representing complete hydrolysis was obtained by subtracting the absorbance at the beginning of the experiment from that after one hour's incubation. The protein concentration of the crude cell extracts and the calculations of specific activity were performed as previously stated in section 2:6 (1).

2:7 Isoelectric focusing

The method used was that originally described by Ambler and Walker (1979) and subsequently employed by Eley *et al.* (1983) to examine the isoelectric points of bacterial β-lactamases.

Sepraphore III 57 x 127 mm cellulose acetate membranes (Gelman Sciences Ltd., Northampton) were passed through increasing concentrations of methanol in water until present in

absolute methanol. The membranes were then methylated by being submerged for one hour at 47°C in 14% boron trifluoride (BDH Chemicals, Poole, Dorset) diluted 2 in 3 with absolute methanol. The treated membranes were then washed and stored in absolute methanol. Before use the treated membranes were equilibrated by soaking for 30 min in a solution of 4% ampholine (Bio-Lyte, pH range 3-10, Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts.) and 7.5% glycerol.

The membranes were then transferred to the glass cooling plate of an LKB 2117 Multiphor (LKB Instruments Ltd., South Croydon, Surrey) and electrode wicks placed in position. The electrode solutions were 3% citric acid at the anode and 1.75% ethanolamine at the cathode, both solutions containing 7.5% glycerol. A constant 400 volts with an initial current of 3.0 mA was then applied across the membrane for 10 minutes. Eight 0.25 µl applications of seven neat or freeze dried concentrated crude β-lactamase extracts, together with a mixture of visible pI marker standards (pH range 4.7-10.6; BDH), were made approximately one third of the way down the membrane from the anode using a double wire applicator (Gelman Sciences). The voltage was reapplied and focusing was complete when the pI markers had reached their equilibrium in the pH gradient, normally within two hours.

Visualization of the β-lactamase bands was achieved by overlaying the membranes with 0.8% agarose containing 250 mg/l nitrocefin. The pI values of the β-lactamases were then determined by comparison with the pI markers.

2:8 Inhibitor profiles

Enzyme inhibition analysis was performed in a centrifugal fast

analyser (Centrifichem 400, Union Carbide, New York, USA) as described initially by Jones *et al.* (1982) and subsequently by Eley and Greenwood (1986b). The colour change induced in nitrocefin at 482 nm as a result of hydrolysis by crude β-lactamase extracts was followed in the presence of serial 10-fold concentrations of β-lactamase inhibitors.

The inhibitors tested were p-chloromercuribenzoate (pcmb) (Sigma), cloxacillin, clavulanic acid, sulbactam, cefoxitin, latamoxef and imipenem at concentrations in the range 0.1-100 μM. The pcmb was initially dissolved in a minimum volume of 1M sodium hydroxide, made up in distilled water and adjusted to pH 7 with 1M hydrochloric acid. The activities of the crude β-lactamase extracts were standardised before testing by appropriate dilution of the neat or concentrated freeze dried extracts with 0.02 M phosphate buffer (pH 7).

The total reaction volume was 300 μl which comprised the following: 250 μl nitrocefin (60 mg/l), 25 μl distilled water, and a mixture of 15 μl crude β-lactamase extract and 10 μl diluted inhibitor. All assays were performed at 37°C after equilibration of the temperature of the reactants for 15 minutes before mixing. Appropriate enzyme and substrate controls were included with each experiment. Reactions were followed for five minutes with readings at one minute intervals. The lowest concentration of inhibitor required to produce 50% or more inhibition of nitrocefin hydrolysis compared to the control, which containined water instead of inhibitor, was defined as the IC_{50}.

2:9 Antibiotic degradation by crude enzyme extracts

The kinetics of hydrolysis by crude β-lactamase extracts of

benzylpenicillin (in the presence of clavulanic acid 4 mg/l), latamoxef, cefoxitin, imipenem, and in later studies meropenem, was investigated by High Pressure Liquid Chromatography (HPLC).

Enzyme extract (0.2 ml) concentrated five times by freeze drying, was mixed in a screw topped reaction vial with 0.8 ml of antibiotic solution to give a final concentration of 50 mg/l and incubated at 37°C in a water bath. Samples of 0.25 ml were removed for HPLC analysis after 0, 2, 4 and 24 hours of incubation. Antibiotic controls, in which phosphate buffer (0.02 M, pH 7) replaced the β-lactamase extract, were included and examined in parallel.

For all analyses, 50 µl of sample was introduced by a Gilson model 231 sample injector (Anachem, Luton, Beds.) onto a reverse-phase separation column packed with Hypersil 5 ODS (HPLC Technology, Macclesfield, Cheshire). The sample, together with the mobile phase, was then pumped at a flow rate of 1 ml/min by means of a Gilson model 302 pump (Anachem). A variable wavelength U.V. absorbance detector (PU 4020; Pye Unicam, Cambridge) was used to detect the presence of antibiotic in the eluate. The mobile phase and detection wavelength for the analysis of each antibiotic is given in Table 2.2.

A series of known antibiotic concentrations was processed to obtain an accurate value for the retention time and peak heights for comparison with sample peaks. From these, the antibiotic concentration in each sample could be calculated.

2.10 Turbidimetry

Continuous opacity records of bacterial growth were obtained by use of the 12-channel Electrotek anaerobic data logging opacity

Table 2.2 HPLC conditions used for β-lactam antibiotic analysis.

Antibiotic	Mobile phase[+]	Detection wavelength (nm)
Benzylpenicillin	methanol[*]:water (40:60)	277
Cefoxitin	methanol[*]:water (40:60)	235
Latamoxef	methanol[*]:water (25:75)	250
Meropenem	methanol[*]:water (25:75)	299
Imipenem	methanol[*]:water (20:80)	299

[+] phosphoric acid (1%) included as mobile phase modifier

[*] HPLC grade (BDH)

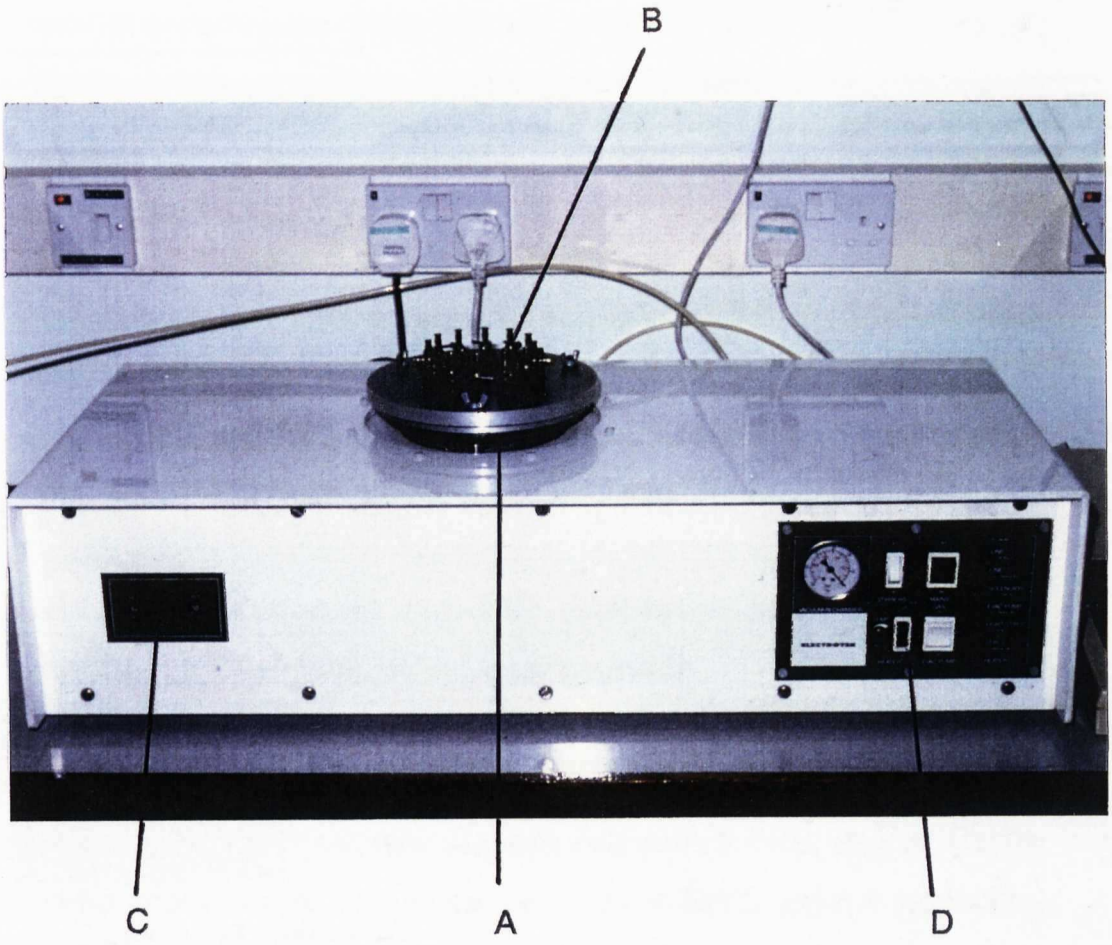
system (Electrotek, Keighley, Yorks.) shown in Fig. 2.1. This comprised an air tight chamber in which a 12 well block was situated. The block could be maintained at a constant temperature of 37°C by an electric heater band. A centrally positioned festoon bulb light source within the block was focused onto photocells by culture tubes present in the wells. A BBC Master series computer examined amplified signals from each channel in turn and these data were simultaneously stored on floppy disc and displayed as percentage opacity against time on a monitor.

The maximum and minimum opacity settings for each channel were adjusted using an uninoculated broth and a fully grown culture. Ten ml volumes of pre-reduced BHIS broth were placed in sterile glass tubes each with a sterile magnetic spinbar (R.B.Radley Ltd., Safron Walden, Essex). They were then inoculated with 0.1 ml of a 1 in 10 diluted overnight culture to give inocula of approximately 10^5 cfu/ml.

For low inoculum experiments, 0.1 ml of an appropriate dilution of antibiotic (or sterile distilled water control) was added to the inoculated tubes to give the required final concentration. Culture tubes were placed in the wells within the chamber. Catalyst envelopes and a GasPak disposable anaerobic indicator (BBL Microbiology Systems, Cockeysville, USA) were then positioned and the lid sealed. The chamber was evacuated and anaerobic gas mixture (80% nitrogen, 10% carbon dioxide, 10% hydrogen) admitted. Evacuation and gas replacement were repeated three times. The stirring mechanism was then turned on and the growth monitored continuously for 30 hours.

To test the response of high inocula, 0.1 ml of a suitable dilution of antibiotic was added to cultures within the chamber when

Fig. 2.1 12-channel Electrotek anaerobic data logging opacity system.



A anaerobic chamber containing 12-well block
B septa to allow passage of fluid into and out of chamber
C adjusters for maximum and minimum opacity settings
D control panel

growth had raised the opacity to 30% of that of a fully grown culture, equivalent to approximately 10^8 cfu/ml. Addition of antibiotic was achieved by the use of syringes which passed through septa in the chamber lid, allowing addition of fluid into the culture tubes without loss of anaerobiosis. Broth cultures could also be withdrawn through these syringes for viable counts, antibiotic assay or morphological examination.

At the end of each experiment, broth from each tube was streaked onto two horse blood agar plates which were incubated aerobically and anaerobically. After 48 hours, the plates were examined to ensure that no contamination of the broth cultures had occurred. Continuous opacity records of bacterial growth were plotted with an 672-XD graph plotter (Hitachi, Tokyo, Japan).

2:11 Degradation of sub-MIC concentrations of antibiotics by growing cultures of resistant strains

Isolates found to exhibit increased resistance to one or more of the test antibiotics were used in this part of the study. One hundred µl of overnight broth cultures of these resistant strains, diluted 1/10 in BHIS broth, were inoculated into 10 ml BHIS broths containing imipenem 0.5 mg/l; latamoxef 16 mg/l; cefoxitin 16 mg/l or benzylpenicillin 0.5 mg/l (with 4 mg/l clavulanic acid). These concentrations were below the MIC for most of the resistant isolates and were detectable in broth residues. After 22 hours of anaerobic incubation at 37°C, the broths were centrifuged and the supernates stored at -70°C. Antibiotic control broths, without bacteria, were also included.

The residual antibiotic was measured by microbiological assay. Indicator organisms were grown for four hours at 37°C in an

appropriate broth and diluted 1/100 in saline. These were a clinical isolate of *Streptococcus pyogenes* for imipenem, *E.coli* NCTC 10418 for latamoxef and the 'Oxford' staphylococcus NCTC 6571 for cefoxitin and benzylpenicillin. The bacterial suspensions were flooded onto Iso-sensitest agar (Unipath) in 20 x 20 cm glass plates and allowed to dry. Wells (7 mm diameter and at least 5 cm apart) were made in the agar and filled with standard dilutions of antibiotic in BHIS or spent broth supernate. After overnight aerobic incubation at 37°C, the zones of inhibition around the wells were measured. A calibration graph was plotted of the square of the zone size against the standard concentrations in $\log_2$ steps, and the concentrations in the test samples were derived by reference to this graph.

2:12 Time course of hydrolysis of sub-MIC concentrations of imipenem by growing cultures of *B.fragilis* strains exhibiting reduced susceptibility to the drug

Ten ml volumes of BHIS broth containing imipenem 0.5 mg/l were seeded with 100 µl of an overnight *B.fragilis* culture in BHIS diluted diluted 1 in 10 with fresh broth. A control without bacteria was also included. These tubes were placed in the Electrotek opacity system and the cultures incubated as described in section 2:10. One ml samples were withdrawn after 0, 6 and 24 hours of incubation and the growth phase at which sampling took place monitored. The culture samples were centrifuged and the amount of imipenem remaining in the supernate was determined by microbiological assay as described in section 2:11.

2:13 Degradation of imipenem (50 mg/l) by whole cells of *B.fragilis* strains exhibiting reduced susceptibility to the drug

Ten ml of BHIS broth cultures of *B.fragilis* strains with reduced susceptibility to imipenem, and *B.fragilis* NCTC 9344, were grown in the Electrotek opacity system in the absence of antibiotic as described for high inoculum experiments in section 2:10. When growth reached 70% of maximum opacity, 0.1 ml of a solution of imipenem was added to achieve a final concentration of 50 mg/l and the experiment allowed to run for a total time of 24 hours. This enabled the imipenem to be in contact with the growing bacteria for approximately 18 hours. After this period the amount of residual imipenem was determined by microbiological assay as described in section 2:11, except that the 'Oxford' staphylococcus was used as the indicator organism for these higher imipenem concentrations and the residue was diluted 1 in 10. Residual imipenem was also measured by HPLC as described in section 2:9. Before chromatography, the broth supernates were treated with acetonitrile (Fisons, Loughborough, Leics.), at a ratio of 1:1, to remove proteins. Antibiotic control broths were also included.

2:14 Determination of inoculum effect

Low and high inoculum experiments were carried out in the Electrotek opacity system as described in section 2:10. *B.fragilis* strains with reduced susceptibility to imipenem were exposed to serial doubling dilutions of the drug. From the growth response curves, MICs and minimum antibacterial concentrations (MACs) were determined and compared. The MIC was defined as the lowest antibiotic concentration required to suppress growth for the whole

period of observation, and the MAC as the lowest antibiotic concentration causing deviation from normal growth.

2:15 Detection of metallo-β-lactamases

(1) With imipenem as substrate

The ability of β-lactamases to hydrolyse imipenem, in the presence and absence of the chelating agent EDTA or EDTA with zinc sulphate or zinc sulphate with phosphate buffer, was determined spectrophotometrically by the methods described by Cuchural *et al.* (1986b), Bandoh *et al.* (1991) and Hedberg *et al.* (1992). One hundred µl of concentrated (x5) crude enzyme extract were mixed with 200 µl EDTA (5 mM) or phosphate buffer (0.02 M; pH 7.0) in a one ml cuvette and incubated at 37°C for 30 minutes. Two hundred µl of either zinc sulphate (5 mM) or phosphate buffer were then added, together with 200 µl imipenem 250 mg/l and a futher 300 µl of phosphate buffer. The absorbance of the imipenem solutions was measured by a spectrophotometer (UV-160A, Shimadzu) at 299 nm after 0, 0.5, 1 and 2 hours incubation at 37°C.

(2) With nitrocefin as substrate

The effect of EDTA, in the presence and absence of zinc sulphate, on the hydrolysis of nitrocefin by crude β-lactamase extracts was examined by the method described above (section 2.15 (1)), except that 200 µl 250 mg/l nitrocefin was added to the cuvette as substrate and the change in absorbance per min was measured over a five minute period at 482 nm and 37°C.

2:16 Effects of clavulanic acid and zinc acetate on broth dilution MICs of imipenem

MICs of imipenem, with and without the β-lactamase inhibitor

clavulanic acid, were determined by the broth dilution method for bacteroides strains with reduced susceptibility to imipenem. Each strain was tested against twofold dilutions of imipenem in 5 ml of BHIS broth, in the presence and absence of clavulanic acid 4 mg/l. Fifty µl of a 1/10 dilution of overnight culture in BHIS broth were added to the antibiotic containing broths which were then incubated anaerobically at 37°C for 48 hours. The MICs were read as the lowest antibiotic concentration required to suppress visible growth.

The impact of increased zinc concentration on the susceptibility of isolates of *B.fragilis* to imipenem in BHIS medium was also assessed. The zinc ion content of BHIS broth was measured using an AA-460 flame atomic absorption spectrophotometer (Perkin Elmer, Beaconsfield, Bucks) in accordance with the manufacturer's instructions and MICs of imipenem were determined in this medium. Sufficient zinc acetate was added to further BHIS broth to double the zinc ion content (from 75 to 150 µmols/l) and the MICs of imipenem were measured again.

2:17 β-Lactamase crypticity measurements

β-Lactamase crypticity was defined as the ratio of the activity observed in preparations of disrupted cells to that of intact cells. The method used was that described by Cuchural *et al.* (1986b) with the degradation of nitrocefin followed spectrophotometrically.

Bacteria from an overnight culture in 10 ml BHIS broth were sedimented by centrifugation and resuspended in 10 ml phosphate buffer (pH 7). This 10 ml suspension was divided into two 5 ml portions, one of which was sonicated to cause cell disruption as described in section 2:5. The rate of hydrolysis of nitrocefin by β-lactamases present in the whole cell suspension and the sonicate

was measured by a spectrophotometer (UV-160, Shimadzu) at 482 nm and 37°C. The reaction mixture present in the cuvette comprised 0.2 ml nitrocefin (250 mg/l), 0.3 ml cell suspension or sonicate and 0.5 ml phosphate buffer (pH 7).

2:18 Outer membrane protein analysis

Outer membrane proteins were examined by a method based on that described by Carlone *et al.* (1986). Overnight cultures of test strains were grown in 20 ml BHIS broth and their optical densities made uniform by the addition of sterile BHIS broth. The cultures were then centrifuged at 4000 rpm for 15 min, the cell pellets washed twice in 50 mM Tris-HCL pH 8 (Sigma) and resuspended in 1 ml Tris-HCL. These were then sonicated on ice with 6 x 30 sec bursts in a Soniprep 150 ultrasonicator (MSE). The sonicates were centrifuged in Eppendorf tubes at 4000 rpm for 15 min to remove any unbroken cells. Each supernate was mixed with 66 µl 30% sodium N-lauroyl sarcosinate (sarkosyl) (BDH), to give a final concentration of 2% (w/v), and incubated at room temperature for 30 min. The mixture was then centrifuged at 13,000 rpm for 20 min in a MicroCentaur microcentrifuge (MSE) and the pellet washed twice in Tris-HCL. The outer membrane proteins present in the pellet were then solubilised by boiling for five minutes with 50 µl Laemmli sample buffer (made up of 4 ml distilled water, 1 ml 0.5 M Tris-HCL pH 6.8, 0.8 ml glycerol, 1.6 ml 10% (w/v) sodium dodecylsulphate (SDS) (Sigma), 0.4 ml 2-b-mercaptoethanol (Sigma) and 0.2 ml 0.05% (w/v) bromophenol blue (BDH)). The samples were centrifuged at 6500 rpm for five minutes to remove any insoluble components and stored at -70°C until protein separation was carried out by SDS-polyacrylamide gel

electrophoresis (PAGE).

SDS-PAGE:

SDS-PAGE analysis was performed with the Mini-PROTEAN II system (Bio-Rad) and a modification of the method described by Laemmli and Favre (1973). The equipment was cleaned thoroughly and assembled according to the instructions provided by Bio-Rad. A gel thickness of 0.75 mm was used. A separating gel of 12% acrylamide was made up from the constituents listed in Table 2.3 and pipetted between two pairs of glass sheets up to 1 cm below the position for the base of the comb. The gels was overlayed with water and allowed to polymerise for one hour. The water was then poured off, and the stacking gel (4% acrylamide, detailed in Table 2.3) pipetted on top of the separating gels with the combs in position. After 30 min, the combs were removed and the gels placed in position on the inner cooling core. Running buffer (Table 2.3) was poured between the gels until the top of the glass plates was covered. Fifteen µl of each sample and 10 µl of a mixture of low range (97 kDa to 14 kDa) molecular weight standards (Bio-Rad) were applied to the gel wells with a P 20 Gilson Pipetman (Anachem). The inner cooling core was placed in an electrophoresis tank and running buffer poured in to cover the bottom of the cooling core. The terminals on the inner cooling core were then connected to a power pack and a constant voltage of 150 v passed. Electrophoresis was allowed to continue until the dye front was 0.5 cm from the bottom of the gel. The gels were then removed from the glass plates and submerged overnight in 0.1% Page Blue 90 stain made up in 40% methanol, 10% acetic acid and 50% distilled water. Destaining was carried out by immersing the gels in 5% methanol, 10% acetic acid and 85% distilled water until the blue background was removed, which normally occurred within

Table 2.3 Constituents of (A) separating gel (B) stacking gel and (C) running buffer used for outer membrane protein analysis

A). Separating gel	12% acrylamide	10.5% acrylamide
Distilled water	3.35 ml	3.85 ml
1.5M Tris-HCL[1] (pH 8.8)	2.5 ml	2.5 ml
10% SDS[1]	100 µl	100 µl
Acrylamide[2] (30%) with (0.03%) N-N bis-methylene-acrylamide[2]	4 ml	3.5 ml
10% Ammonium persulphate[1] (freshly prepared)	50 µl	50 µl
TEMED[3]	10 µl	10 µl

B). Stacking gel (4% acrylamide)	
Distilled water	6.1 ml
0.5M Tris-HCL (pH 6.8)	2.5 ml
10% SDS	100 µl
Acrylamide (30%) with (0.03%) N-N bis-methylene-acrylamide	1.3 ml
10% Ammonium persulphate (freshly prepared)	50 µl
TEMED	15 µl

C). Running buffer (x5 conc.) pH 8.3	
Tris base[3]	7.5g
Glycine[2]	36g
SDS	2.5g
Distilled water	500 ml

Suppliers: [1] Sigma Chemical Co. [2] BDH Chemicals
[3] Bio Rad Laboratories Ltd

one hour. The gels were photographed and the molecular sizes of protein bands calculated by comparison with the standards.

2:19 Lipopolysaccharide analysis

Lipopolysaccharides (LPS) present in the outer membrane were investigated by a method developed by Hitchcock and Brown (1983). Bacteria were harvested from 10 ml overnight BHIS broth culture by centrifugation at 4,000 rpm for 15 min, and washed once in phosphate buffered saline (PBS). They were then suspended in PBS to give spectrophometric readings of between 0.5 and 0.6 at 525 nm. Bacteria from 1.5 ml of this suspension were sedimented in a MicroCentaur microcentrifuge (MSE) at 6500 rpm for five minutes. The cell pellet was mixed with 0.05 ml Laemmli sample buffer (detailed in section 2:18) and heated at 100°C for 10 min. Ten µl of a solution of 5 mg proteinase K (Sigma) in 2 ml Laemmli sample buffer were added, and the mixture incubated at 60°C for one hour. The lipopolysaccharides were separated by electrophoresis as described in section 2:18, except that the separating gels were composed of 14% polyacrylamide without SDS as follows: 2.68 ml distilled water, 2.5 ml Tris-HCL pH 8.8, 4.66 ml 30% acrylamide with 0.03% N-N bis-methylene-acrylamide, 50 µl 10% ammonium persulphate and 10 µl TEMED. SDS was also omitted from the stacking gels.

The separating gels were stained by silver stain following the method of Hancock and Poxton (1988). The gels were fixed overnight in 200 ml 25% propan-2-ol (Fisons) and 7% acetic acid. They were oxidised for five minutes in a freshly prepared solution of 1.05 g periodic acid (Sigma) in 150 ml distilled water containing 4 ml of the propan-2-ol fixative, and washed in four changes of 200 ml distilled water over a four hour period. A solution of ammoniacal silver nitrate

was then freshly prepared as follows: 1.4 ml ammonia solution (specific gravity 0.88) was added to 21 ml 0.36% sodium hydroxide and 4 ml of 19.4% silver nitrate (Sigma) solution was slowly added with vigorous agitation. The final volume was made up to 100 ml with distilled water. The gels were stained in ammoniacal silver nitrate for 15 min, removed from the silver solution and washed four times in 200 ml distilled water over a period of 40 min. They were then soaked in fresh 0.005% citric acid in 200 ml of 0.019% formaldehyde at 25°C for 15 min, and washed repeatedly with distilled water. The gels were photographed and the LPSs of each strain compared.

2:20 Analysis of penicillin binding proteins (PBPs)

The method used was based on the procedure described by Spratt (1977a). BHIS broth (200 ml) was inoculated with 7.5 ml of an overnight culture of the test strain in BHIS broth. After four hours of anaerobic incubation at 37°C, the bacteria were harvested by centrifugation at 4,000 rpm for 15 min. They were washed once and resuspended in 10 ml phosphate buffer (0.02 M, pH 7.0). The bacterial suspension was sonicated on ice for 6 x 30 sec in a Soniprep 150 ultrasonicator (MSE), then centrifuged at 4,000 rpm for 15 min to remove the unbroken cells. The supernate was centrifuged at 40,000 rpm and 4°C for 30 min in a Model L5-65 ultracentrifuge (Beckman, High Wycombe, Bucks.) to sediment the cell membranes which were then washed in phosphate buffer five times. The pellet was resuspended in 2 ml of phosphate buffer and the protein content measured with a Sigma Protein Assay Kit (Sigma) in accordance with the manufacturer's instructions. The suspension was centrifuged to pellet the cell membranes which were then reconstituted in

phosphate buffer to achieve a protein concentration of 10 mg/ml. The sample was tested for the presence of penicillinase by the method of Escamilla (1976) (See section 2:3), divided into 50 µl portions and stored at -70°C until use.

Twenty µl of cell membrane suspension and 5µl distilled water were pre-warmed to 30°C. Five µl of ^{3}H-benzylpenicillin (Amersham International plc, Aylesbury, Bucks.), with a radioactive concentration of 1.0 mCi/ml and a benzylpenicillin concentration of 18.8 mg/l (final penicillin concentration 3.1 mg/l and a radioactive content of 5 µCi), were added and the mixture left for 10 min at 30°C. Excess non-radioactive penicillin was introduced by adding 5 µl benzylpenicillin at a concentration of 120 mg/l. Thirty-five µl of double strength Laemmli sample buffer (see section 2:18) was added and the mixture boiled for five minutes to solubilise the proteins present. The sample was then divided into 17.5 µl portions and stored at -70°C. In later experiments, 15 µl and 30 µl of ^{3}H-benzylpenicillin were used with a corresponding increase in volume of double strength sample buffer. Also, the 5 µl volumes of distilled water added to the cell membrane suspension were replaced by clavulanic acid or sulbactam to give final concentrations of 5 mg/l and 10 mg/l respectively and exposed to 10 µl ^{3}H-benzypenicillin. For competition assays, 5 µl volumes of doubling dilutions of imipenem were used instead of distilled water, together with 5 µl ^{3}H-benzylpenicillin.

The cell membrane proteins were then separated on SDS-PAGE gels as described in section 2:18. Separating gels containing 10.5% acrylamide were used with 4% acrylamide present in the stacking gels (See table 2.3). The gels were either divided into segments which were examined by scintillation counter or analysed by fluorography.

(1) Scintillation counts of gel segments

Each protein track was cut into two or more segments representing known molecular weight ranges by reference to the molecular weight markers. Each gel segment was then placed in a glass vial with 3 ml scintillation fluid made by mixing 0.1g 1,4-Di-[2-(5-phenyloxazoyl)]-benzene (Koch-Light Ltd, Hatfield, Herts.) and 3g 2, 5-Di phenyloxazole (Koch-Light) in one litre of low sulphur grade toluene (Fisons). After ten days, the level of radioactivity was counted in a SL 3000 liquid scintillation counter (Kontron, Watford, Herts.). Segments from an area of gel away from the sample tracks were processed in a similar fashion to provide a count of the background radioactivity.

(2) Fluorography

The water used for storing the stained gel was poured off and, in the fume cupboard, enough En(^{3}H)ance (NEN Research Products, Boston, USA) was added to cover the gel. After one hour the En(^{3}H)ance was poured off and the gel covered with deionized water and left for a further hour. The water was then replaced with fresh deionized water. The gel was then removed, placed on a sheet of filter paper, covered with cling film and dried at 80°C under vacuum on a model 443 slab drier (Bio-Rad). The dried gel was taken into the dark room where all procedures were carried out under safe light. A Kodak X-OMAT XAR 5 film (Sigma) was pre-fogged by use of a flash gun. The intensity of the flash was reduced by placing the flash gun inside a black bag with a small hole and aiming it at the ceiling of the dark room. The gel was placed on the pre-fogged side of the film, and the gel and film sandwiched between two aluminium plates which were then placed in an exposure cassette (Sigma). The cassette was then sealed inside two black plastic bags and stored at -70°C. After

three weeks (seven weeks in later experiments) the cassette was returned to the dark room and, under safe light, the film removed after the position of the gel had been recorded by small cuts on the side of the film. The film was then developed by immersion in a 1 in 10 dilution of PQ Universal Developer (Ilford, Mobberley, Cheshire) for 6 min, followed by a 1 in 40 dilution of acetic acid for 10 sec and then Ilfospeed fixer (Ilford) diluted 1 in 4 for 2 min. After washing the film, the position of the dark bands, indicating emitted radioactivity of PBPs, could be measured and, by reference to the position of the protein markers on the gel, the molecular weight of the radioactive proteins determined.

CHAPTER 3

IDENTIFICATION, ANTIBIOTIC SUSCEPTIBILITY AND β-LACTAMASE PRODUCTION OF CLINICAL ISOLATES OF BACTEROIDES.

CHAPTER 3

IDENTIFICATION, ANTIBIOTIC SUSCEPTIBILITY AND β-LACTAMASE PRODUCTION OF CLINICAL ISOLATES OF BACTEROIDES

3:1 Introduction

Clinically significant bacteroides strains, isolated at the Nottingham Public Health Laboratory during 1989 and 1990, were investigated to determine their species, susceptibility to various β-lactam antibiotics and degree of β-lactamase production. These findings were compared with those of previous studies. The degree of resistance to β-lactam antibiotics was assessed to detect any increase in resistance since the report of a similar study in Nottingham was published in 1986 (Eley and Greenwood, 1986a). The extent of the correlation between antibiotic resistance and β-lactamase production was also examined. Isolates exhibiting increased resistance to β-lactam antibiotics were identified and used for subsequent studies into their mechanisms of resistance.

3:2 Identification of isolates

Of the 121 anaerobic Gram negative bacilli collected, 108 were found to belong to the *Bacteroides* genus, and were used in the study. They were identified as follows: *B.fragilis* (69), *B.distasonis* (16), *B.ovatus* (13), *B.thetaiotaomicron* (6) and *B.vulgatus* (4).

3:3 Antibiotic titrations

MICs of the antibiotics for all 108 test strains are summarised in

Table 3.1. MIC_{90} values for imipenem and for benzylpenicillin in the presence of clavulanic acid (4 mg/l) were 1 and 2 mg/l respectively, substantially lower than those of latamoxef, cefoxitin or benzylpenicillin alone (MIC_{90}: 16->64 mg/l).

3:4 Semi-quantification of β-lactamase activity in intact bacteria

Of the 108 isolates tested, 82 (76%) produced β-lactamase (cephalosporinase) as indicated by the induction of a colour change in the chromogenic cephalosporin nitrocefin. Sixty-one strains (57%) produced a slight colour change and 21 (19%) produced elevated β-lactamase levels causing a complete colour change within one hour. The remaining 26 strains failed to induce a colour change. The 21 strains which produced elevated levels of cephalosporinase comprised 14 *B.fragilis*, 5 *B.ovatus*, 1 *B.thetaiotaomicron* and 1 *B.distasonis* (Table 3.2).

Fifteen (14% of all strains examined) of these 21 strains also gave a positive reaction in the phenol red test for penicillinase, although two of these strains produced only a weak reaction. Nine of the 12 strains which produced a rapid and complete colour change in the nitrocefin cephalosporinase test elicited a rapid reaction in the phenol red test for penicillinase production.

Further studies were restricted to these 21 isolates of bacteroides which produced raised amounts of β-lactamase.

3:5 Antibiotic titrations for strains with raised β-lactamase levels

Results of antibiotic titrations for the 21 strains with raised β-

Table 3.1 Susceptibilities of 108 isolates of bacteroides to selected β-lactam antibiotics

Antibiotic	Minimum inhibitory concentration (mg/l) Range	MIC_{50}	MIC_{90}
Benzylpenicillin	0.5->64	16	>64
Benzylpenicillin + clavulanic acid*	0.06-16	0.25	2
Cefoxitin	0.25->128	16	32
Latamoxef	0.25->128	1	16
Imipenem	0.03-4	0.5	1

MIC_{50} and MIC_{90}, concentrations inhibiting 50% and 90% of strains respectively

*Clavulanic acid at fixed concentration of 4 mg/l

Table 3.2 Semi-quantification of cephalosporinases and penicillinases in intact bacteroides with raised β-lactamase activity.

Bacterial strain	cephalosporinase (nitrocefin test)	penicillinase (phenol red test)
B.fragilis R106	+++	-
B.fragilis R130	++	-
B.fragilis R249	++	-
B.fragilis R251	+	++
B.fragilis R186	+++	-
B.fragilis R240	++	++
B.ovatus R137	++	++
B.ovatus R111	+++	+
B.distasonis R118	++	+
B.fragilis R208	++	-
B.fragilis R141	++	-
B.fragilis R126	+++	+++
B.ovatus R215	++	+++
B.fragilis R152	+++	+++
B.ovatus R112	+++	+++
B.fragilis R226	+++	+++
B.fragilis R216	+++	+++
B.ovatus R102	+++	+++
B.fragilis R212	+++	+++
B.fragilis R134	+++	+++
B.thetaiotaomicron R233	+++	+++
B.fragilis NCTC 9344*	+/-	-

-,+/-,+,++,+++ indicates levels of enzyme activity assessed visually according to the speed and completeness of the colour change.
* control strain

lactamase levels are displayed in Table 3.3. Fourteen of these strains exhibited increased resistance to benzylpenicillin (MIC ≥32 mg/l). Clavulanic acid (4 mg/l) reduced the MIC of benzylpenicillin more than two-fold for all but three of the 21 strains (*B.fragilis* R186, R240 and R249) and resulted in MICs of benzylpenicillin of <2 mg/l for all but six of the strains (*B.fragilis* R186, R212, R233, R240, R249 and R251). A wide range of susceptibilities to the other test antibiotics was displayed. Nine strains showed an increase in resistance to cefoxitin (MIC ≥32 mg/l), latamoxef (MIC ≥32 mg/l) or imipenem (MIC ≥2 mg/l) with MICs of at least two of these antibiotics greater than or equal to their corresponding MIC_{90}. Three of these strains, *B.fragilis* R208, *B.fragilis* R212 and *B.thetaiotaomicron* R233, showed reduced susceptibility to cefoxitin, latamoxef and imipenem. Four strains, *B.fragilis* R251, *B.fragilis* R186, *B.fragilis* R240 and *B.distasonis* R118, showed increased resistance to imipenem and cefoxitin. Also, *B.ovatus* R215 showed marked resistance to cefoxitin and latamoxef and the MICs of these antibiotics for *B.ovatus* R102 were raised.

This group of 21 high level β-lactamase producers contained all the strains which were resistant to the test antibiotics with the exception of three other bacteroides isolates (2 *B.distasonis* and 1 *B.thetaiotaomicron*) for which the MIC of cefoxitin was 32 mg/l; these isolates were susceptible to latamoxef and imipenem. The remaining 84 isolates were sensitive to the test antibiotics. (MICs of cefoxitin and latamoxef <32 mg/l; MIC imipenem <2 mg/l).

Therefore, 12 of the 108 isolates tested (11% of strains) showed increased resistance to cefoxitin. Of these, nine (8%) produced elevated amounts of β-lactamases; two of these strains (2%) showed high resistance levels (MIC cefoxitin >64 mg/l). Five isolates (5%) were resistant to latamoxef and seven (6%) showed

Table 3.3 Antibiotic susceptibility (MIC mg/l) of 21 strains of *Bacteroides* that exhibited increased β-lactamase activity

Bacterial strain	Minimum inhibitory concentration (mg/l)				
	benzyl penicillin	benzylpenicillin +clavulanic acid#	cefoxitin	latamoxef	imipenem
B.fragilis R106	32	0.5	16	1	0.5
B.fragilis R130	32	0.5	8	1	0.25
B.fragilis R249	4	2	16	0.5	1
B.fragilis R251	16	2	32	4	2
B.fragilis R186	8	4	32	1	2
B.fragilis R240	8	4	32	1	4
B.ovatus R137	16	0.5	8	1	0.5
B.ovatus R111	16	0.25	4	1	0.5
B.distasonis R118	32	1	32	16	2
B.fragilis R208	>64	1	32	>128	4
B.fragilis R141	16	0.25	16	0.5	0.06
B.fragilis R126	>64	1	16	16	0.5
B.ovatus R215	>64	0.5	64	>128	1
B.fragilis R152	>64	<0.12	8	8	0.12
B.ovatus R112	>64	0.25	16	16	0.5
B.fragilis R226	>64	0.5	8	16	1
B.fragilis R216	>64	1	8	8	1
B.ovatus R102	>64	1	32	32	0.5
B.fragilis R212	>64	4	128	>128	2
B.fragilis R134	>64	0.25	16	16	0.25
B.thetaiotaomicron R233	>64	16	>128	>128	4
B.fragilis NCTC 9344*	16	0.25	8	0.25	0.12

* control strain

\# clavulanic acid at a fixed concentration of 4 mg/l

reduced susceptibility to imipenem. These strains all produced elevated amounts of β-lactamase and were cefoxitin resistant. Four of the latamoxef resistant strains were highly resistant (MICs >64 mg/l), whereas none of the strains showing raised MICs to imipenem were highly resistant (MIC >64 mg/l).

3:6 Discussion

Modest inconsistancies are apparent between this study and previous reports in terms of the types of species of *Bacteroides* isolated from clinical samples and the degree of β-lactamase production by these strains. These discrepancies may have been due to differences in the clinical material examined, technical procedures, evaluation of test results or simply natural variation.

Most (89%) anaerobic Gram negative bacilli isolated from clinical material in this study were identified as *Bacteroides* species, which is consistant with previous studies (Holland *et al.*, 1977). *B.fragilis*, the predominant species, comprised 57% of the of the clinically significant anaerobic Gram negative bacilli and 64% of the bacteroides, reflecting this organism's pathogenic potential and virulence properties. These findings are similar to those of Duerden (1980a), who reported that 51% of all isolates of anaerobic Gram negative bacilli and 78% of the 'fragilis group' were *B.fragilis*. Eley and Greenwood's Nottingham study in 1986, however, described 60 of their 78 clinical isolates (77%) as *B.fragilis*, the remainder belonging to the 'fragilis group' (Eley and Greenwood, 1986a).

Duerden (1980a) and Eley and Greenwood (1986a) found *B.thetaiotaomicron* to be the second most common *Bacteroides* species representing 13% and 20% of the bacteroides isolated

respectively. However, in this study, *B.thetaiotaomicron* only comprised 6% of the bacteroides strains. The most common *Bacteroides* species after *B.fragilis* were *B.distasonis* and *B.ovatus*, comprising 15% and 12% of the isolates respectively.

Imipenem and the combination of benzylpenicillin with clavulanic acid were shown to be considerably more active than the other β-lactam antibiotics tested, as judged by conventional MIC titrations. These findings are comparable with the results of the study by Eley and Greenwood (1986a) and with recent reports from Europe and USA (Betriu *et al.*, 1992; Dubreuil *et al.*, 1992; Phillips *et al.*, 1992; Goldstein *et al.*, 1993).

Most clinical strains of bacteroides (76%) have been shown to produce β-lactamases which are predominately cephalosporinases. Olsson *et al.* (1977) described the production of small amounts of β-lactamase by most bacteroides isolates (87%), with 6% of strains forming elevated amounts of the enzyme. More recently, Aldridge *et al.* (1988) found that 97% of 246 *B.fragilis* isolates from the United States produced β-lactamases. Eley and Greenwood (1986a) reported that 74 of the 78 (95%) clinical isolates from Nottingham showed β-lactamase activity when nitrocefin was used as substrate, 13 (17%) of these were shown to produce elevated levels of the enzyme. Other reports on β-lactamase-producing *B.fragilis* give frequencies of 50% and 90%, depending on the sensitivity and type of assay used and the definition used to designate positive test results (Darland and Birnbaum, 1977; Montgomery *et al.*, 1979). Eley and Greenwood (1986a) reported that ten (13%) out of the 78 clinical isolates from Nottingham gave positive reactions in the phenol red test for penicillinase, similar to the findings of this study where 14% of strains gave positive reactions with benzylpenicillin as substrate.

Eley and Greenwood (1986a) classified all but one of the 13 'high β-lactamase' producers as *B.fragilis*. This is in contrast to the findings of this study, where only 14 of the 21 strains which produced elevated levels of cephalosporinase were identified as *B.fragilis*.

The correlation between elevated β-lactamase production and β-lactam antibiotic resistance, described many years ago by Anderson and Sykes (1973) and Olsson *et al.* (1977), was also observed in this present study. The 14 isolates showing high level resistance to benzylpenicillin, and nine of the twelve strains with increased resistance to cefoxitin which included those with increased resistance to latamoxef or imipenem or both, belonged to the group of 21 strains with elevated β-lactamase levels. These findings reinforce the notion that β-lactamases are a major factor in the resistance of *Bacteroides* species to β-lactam antibiotics (Rasmussen *et al.*, 1993). Also, benzylpenicillin in combination with clavulanic acid has been shown to be highly active against clinical isolates of bacteroides. The β-lactamase inhibitor restored high activity to benzylpenicillin in all but six *B.fragilis* strains indicating the importance of clavulanic acid sensitive β-lactamases in bacteroides resistance. However, three isolates, that did not produce elevated levels of β-lactamase, showed low resistance to cefoxitin, suggesting the role of resistance factors other than β-lactamases.

The high level resistance to latamoxef and cefoxitin detected in the present study appeared marginally more prevalant than that described by Eley and Greenwood (1986a) who found no highly cefoxitin resistant bacteroides, although the MIC of cefoxitin for 4% of the total number of strains tested was 32 mg/l. Each of these strains exhibited increased β-lactamase activity. These authors reported 6% and 4% of the total number of test strains as resistant to latamoxef and

imipenem respectively. Only one out of the five isolates for which the MIC of latamoxef was raised was highly resistant to the antibiotic, and, as in the present study, no highly imipenem resistant strains were reported. Highly imipenem resistant strains have been occasionally isolated in other centres. Cuchural *et al.* (1986b) found two out of over 350 isolates tested in Boston, USA, to be highly resistant to imipenem, and other sporadic isolates have been reported from Japan, France and Sweden (Bandoh *et al.*, 1991; Podglajen *et al.*, 1992a; Hedberg *et al.*, 1992).

The extent of resistance to the 'β-lactamase stable' β-lactams cefoxitin, latamoxef and imipenem in Nottingham appears low and has not increased in the intervening five years since isolates were collected for the Eley and Greenwood (1986a) study, with the exception of a modest increase in cefoxitin resistance. Interestingly, a lower cefoxitin resistance rate (3%) compared to the present study, which was similar to that reported by Eley and Greenwood (1986a), was found Phillips *et al.* (1992) in a European study. These findings are reassuring in that they do not expose a rapidly increasing resistance problem associated with this group of organisms and antibiotics.

CHAPTER 4

CHARACTERISATION AND CLASSIFICATION OF BACTEROIDES β-LACTAMASES

CHAPTER 4

CHARACTERISATION AND CLASSIFICATION OF BACTEROIDES β-LACTAMASES

4:1 Introduction

Characterisation of β-lactamases produced by *Bacteroides* species has traditionally resulted from the investigation of physical and chemical properties including specific activity, isoelectric point, inhibitor profiles, molecular weight and hydrolysis capability (Leung and Williams, 1978; Nord and Olsson-Liljequist; 1981; Timewell *et al.*, 1981). By use of these features, typical *B.fragilis* β-lactamases have been characterised and categorised (Britz and Wilkinson, 1978; Bush, 1989c). Other diverse β-lactamase types from *Bacteroides* species have been described and attempts to classify them have been made. (Sato *et al.*, 1980, 1982; Cuchural *et al.*, 1983, 1986b; Yotsuji *et al.*, 1983; Eley and Greenwood, 1986a,b; Bandoh *et al.*, 1991; Hedberg *et al.*, 1992). In this part of the study, β-lactamases from the 21 strains which produced elevated amounts of the enzyme were examined. Their characteristics were classified and compared to those of other studies.

4:2 Specific cephalosporinase activity

Specific cephalosporinase activity (μmoles of nitrocefin degraded/min/mg of protein) of the crude enzyme extracts ranged from 0.01 to 1.33 (Table 4.1). These values were generally lower than those obtained for the 13 clinical isolates of bacteroides described by Eley and Greenwood (1986a), who used similar methods with

Table 4.1 Specific activities of isolates of bacteroides that exhibited increased β-lactamase activity.

Bacterial strain	Specific activity[#]
B.fragilis R106	0.01
B.fragilis R130	0.01
B.fragilis R249	0.01
B.fragilis R251	0.01
B.fragilis R186	0.02
B.fragilis R240	0.02
B.ovatus R137	0.03
B.ovatus R111	0.05
B.distasonis R118	0.07
B.fragilis R208	0.08
B.fragilis R141	0.09
B.fragilis R126	0.15
B.ovatus R215	0.16
B.fragilis R152	0.21
B.ovatus R112	0.44
B.fragilis R226	0.68
B.fragilis R216	0.71
B.ovatus R102	0.87
B.fragilis R212	0.88
B.fragilis R134	0.93
B.thetaiotaomicron R233	1.33
B.fragilis NCTC 9344*	0.003

*control strain

[#]μmoles nitrocefin degraded/min/mg of protein

nitrocefin as substrate. These differences may be due to the different population of organisms under investigation. Also, it has been shown that variations in the conditions of growth and time of harvesting of the organisms, and concentration and storage of the enzyme extracts, may profoundly affect the results (Olsson *et al.*, 1976; Edwards and Greenwood, 1990). Comparison of specific activity with data present in other reports is difficult due to differences in methods and substrate used.

As expected, good correlation was shown between the specific activity and whole cell cephalosporinase and penicillinase tests (See section 3.4). All the strains with specific activity of more than 0.2 exhibited rapid and complete penicillinase and cephalosporinase activity as determined by the whole cell semi-quantitative tests. None of the isolates with specific activity of less than 0.1 showed high penicillinase activity and these included six bacteroides isolates in which no penicillinase was detected. Four of the 13 strains with specific activity of less than 0.2 displayed high whole cell cephalosporinase activity.

4:3 Isoelectric focusing

The isoelectric points of β-lactamases present in crude extracts of bacteroides isolates with elevated β-lactamase levels are listed in Table 4.2. Most *B.fragilis* strains yielded a single isoelectric point between 4.9 and 5.3. No band was detected in extracts of three other *B.fragilis* strains (R240, R249 and R251) despite repeated testing. Two strains, *B.distasonis* R118 and *B.ovatus* R215, possessed enzymes which focused at two isoelectric points. Enzymes from the five *B.ovatus* strains displayed a wide range of pI values (4.5-6.7).

These findings are in broad agreement with previous reports.

Table 4.2 Isoelectric points (pI values) of β-lactamases from isolates of bacteroides with elevated β-lactamase levels.

Bacterial strain	Isoelectric point
B.fragilis R106	5.3
B.fragilis R130	5.0
B.fragilis R249	ND
B.fragilis R251	ND
B.fragilis R186	4.9
B.fragilis R240	ND
B.ovatus R137	4.5
B.ovatus R111	4.8
B.distasonis R118	4.6,5.0
B.fragilis R208	5.0
B.fragilis R141	5.0
B.fragilis R126	5.0
B.ovatus R215	5.0,6.7
B.fragilis R152	5.2
B.ovatus R112	5.1
B.fragilis R226	4.9
B.fragilis R216	4.9
B.ovatus R102	5.0
B.fragilis R212	5.1
B.fragilis R134	4.9
B.thetaiotaomicron R233	4.6
B.fragilis NCTC 9344*	5.1

ND not detected
* control strain

Dornbusch *et al.* (1980) described an isoelectric point of 4.9 for 20 isolates of *B.fragilis*. Diverse pI values for *B.ovatus* β-lactamases, 4.0-7.1, have also been reported (Leung and Williams, 1978; Dorndusch *et al.*, 1980; Timewell *et al.*, 1981). Leung and Williams (1978) and Tally *et al.* (1979) gave isoelectric points for *B.distasonis* of 5.1 and 4.8, and for *B.thetaiotaomicron* 4.5 and 4.6 respectively, similar to the results of this study.

4:4 Inhibitor profiles

Results of experiments designed to test the ability of p-chloromercuribenzoate (pcmb) and various antimicrobial compounds to inhibit enzyme activities are shown in Table 4.3.

The concentrations of inhibitors required to achieve inhibition of the individual enzymes varied considerably. There were only two profiles common to more than one strain. These were those shown by *B.fragilis* R130 and *B.fragilis* R152, and *B.ovatus* R137 and *B.fragilis* R141. Five of the 21 strains, (*B.fragilis* R106, *B.fragilis* R216, *B.fragilis* R226, *B.ovatus* R111 and *B.ovatus* R112) produced β-lactamases which were at least partially susceptible to all the inhibitors tested, and a further seven isolates were susceptible to some degree to all but pcmb.

The β-lactamase from *B.fragilis* R186 was not inhibited by any of the agents tested, and those from *B.fragilis* R240, *B.fragilis* R249 and *B.fragilis* R251 displayed very low levels of susceptibilty with no inhibition by imipenem, latamoxef, cefoxitin, clavulanic acid or sulbactam. All of the remaining 17 strains possessed β-lactamases which were fully susceptible to inhibition by imipenem. Four of these enzymes showed some degree of inhibition by all of the four compounds latamoxef, cefoxitin, clavulanic acid and sulbactam

Table 4.3 Inhibitor profiles of β-lactamases of 21 strains of *Bacteroides* that exhibited increased β-lactamase activity

Bacterial strain	IC_{50} (μM)						
	pcmb	imipenem	latamoxef	cefoxitin	clavulanic acid	sulbactam	cloxacillin
B.fragilis R106	10	<0.1	<0.1	<0.1	<0.1	<0.1	10
B.fragilis R130	>100	<0.1	<0.1	<0.1	<0.1	1	100
B.fragilis R249	10	>100	>100	>100	>100	>100	100
B.fragilis R251	100	>100	>100	>100	>100	>100	100
B.fragilis R186	>100	>100	>100	>100	>100	>100	>100
B.fragilis R240	100	>100	>100	>100	>100	>100	>100
B.ovatus R137	>100	<0.1	<0.1	<0.1	<0.1	<0.1	100
B.ovatus R111	10	<0.1	<0.1	<0.1	<0.1	<0.1	100
B.distasonis R118	>100	<0.1	<0.1	<0.1	1	10	>100
B.fragilis R208	>100	<0.1	100	1	10	10	>100
B.fragilis R141	>100	<0.1	<0.1	<0.1	<0.1	<0.1	100
B.fragilis R126	>100	<0.1	1	<0.1	<0.1	<0.1	100
B.ovatus R215	>100	<0.1	10	1	1	1	100
B.fragilis R152	>100	<0.1	<0.1	<0.1	<0.1	1	100
B.ovatus R112	100	<0.1	<0.1	<0.1	<0.1	<0.1	100
B.fragilis R226	1	<0.1	<0.1	<0.1	<0.1	<0.1	10
B.fragilis R216	1	<0.1	<0.1	<0.1	<0.1	<0.1	1
B.ovatus R102	100	<0.1	<0.1	<0.1	<0.1	>100	>100
B.fragilis R212	>100	<0.1	100	1	1	1	>100
B.fragilis R134	>100	<0.1	<0.1	1	<0.1	<0.1	100
B.thetaiotaomicron R233	>100	<0.1	100	1	1	10	>100
B.fragilis NCTC 9344*	10	<0.1	<0.1	<0.1	<0.1	<0.1	10

IC_{50}, lowest concentration of inhibitor producing >50% inhibition

pcmb, p-chloromercuribenzoate

* control strain

(*B.fragilis* R208, *B.ovatus* R215, *B.fragilis* R212 and *B.thetaiotaomicron* R233) and the β-lactamase from *B.distasonis* R118 was not fully susceptible to clavulanic acid and sulbactam.

Typical *B.fragilis* β-lactamases have been shown to be inhibited by clavulanic acid and pcmb (Timewell *et. al.*, 1981; Yotsuji *et al.*, 1983). Eley and Greenwood (1986a) described inhibitor profiles of clinical bacteroides isolates by similar methods and inhibitors to those used in this study. Of 13 strains studied, seven were at least partially susceptible to all inhibitors tested, and a further three were susceptible to some degree to all inhibitors except pcmb. Two other strains showed overall low levels of susceptibility identical to that displayed by *B.fragilis* R240 in this study.

4:5 Antibiotic hydrolysis by crude β-lactamase extracts

Hydrolysis of the β-lactam antibiotics was assessed by HPLC. Concentrated crude enzyme extracts from nine of the 21 isolates of bacteroides producing elevated amounts of β-lactamase were able to hydrolyse benzylpenicillin (in the presence of clavulanic acid), cefoxitin, latamoxef or imipenem over the 20-hour incubation period (Figs. 4.1-4.4). These nine strains included the eight isolates showing increased resistance to cefoxitin, latamoxef or imipenem in MIC titrations (See section 3:5) and *B.fragilis* R249 which was of intermediate susceptibility to cefoxitin and imipenem (MICs of 16 mg/l and 1 mg/l respectively), and susceptible to latamoxef (MIC 0.5 mg/l) (See table 3.3). Extracts of the other resistant strain, *B.ovatus* R102, which showed low level resistance to cefoxitin and latamoxef (MICs of 32 mg/l for both antibiotics), failed to hydrolyse any of the β-lactam antibiotics tested.

Fig. 4.1 Hydrolysis of benzylpenicillin in the presence of clavulanic acid 4 mg/l by enzyme extracts from bacteroides strains

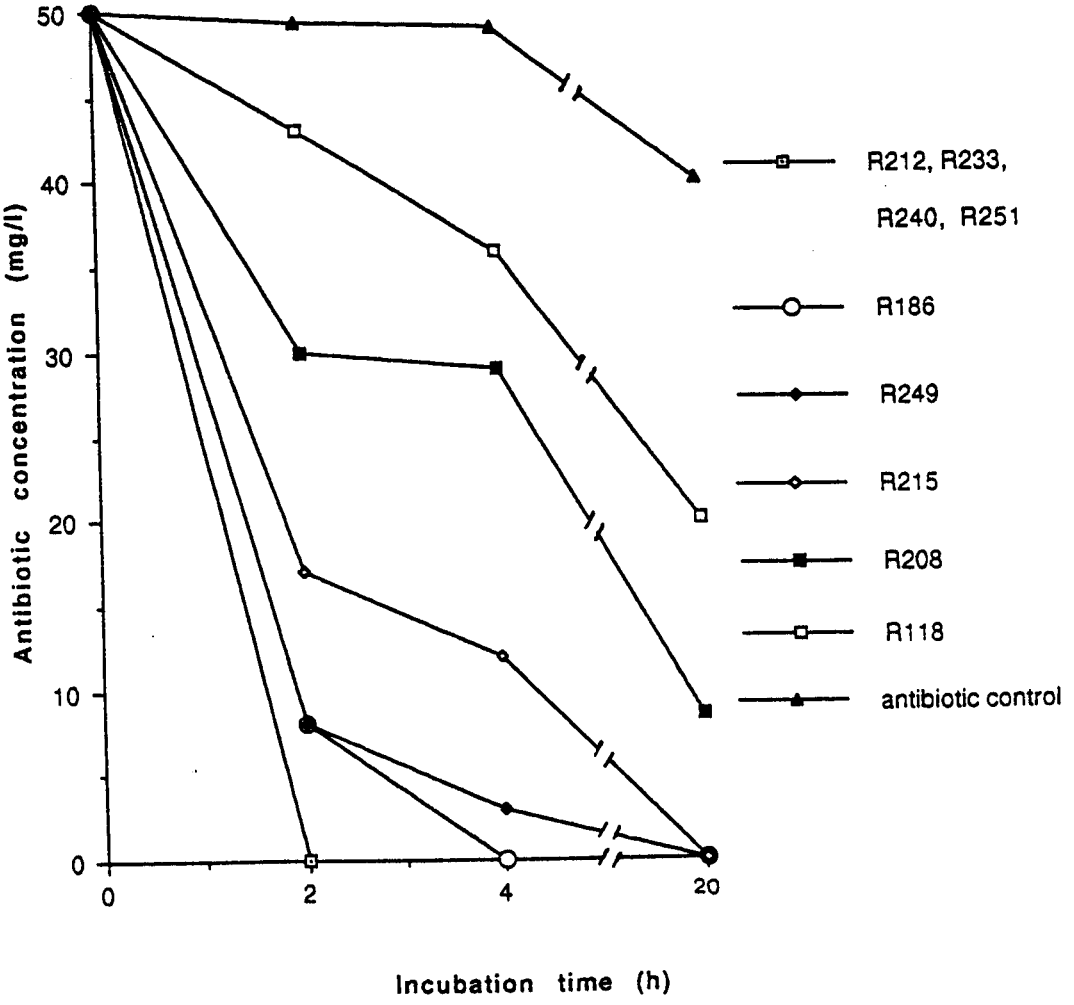


Fig. 4.2 Hydrolysis of cefoxitin by enzyme extracts from bacteroides strains

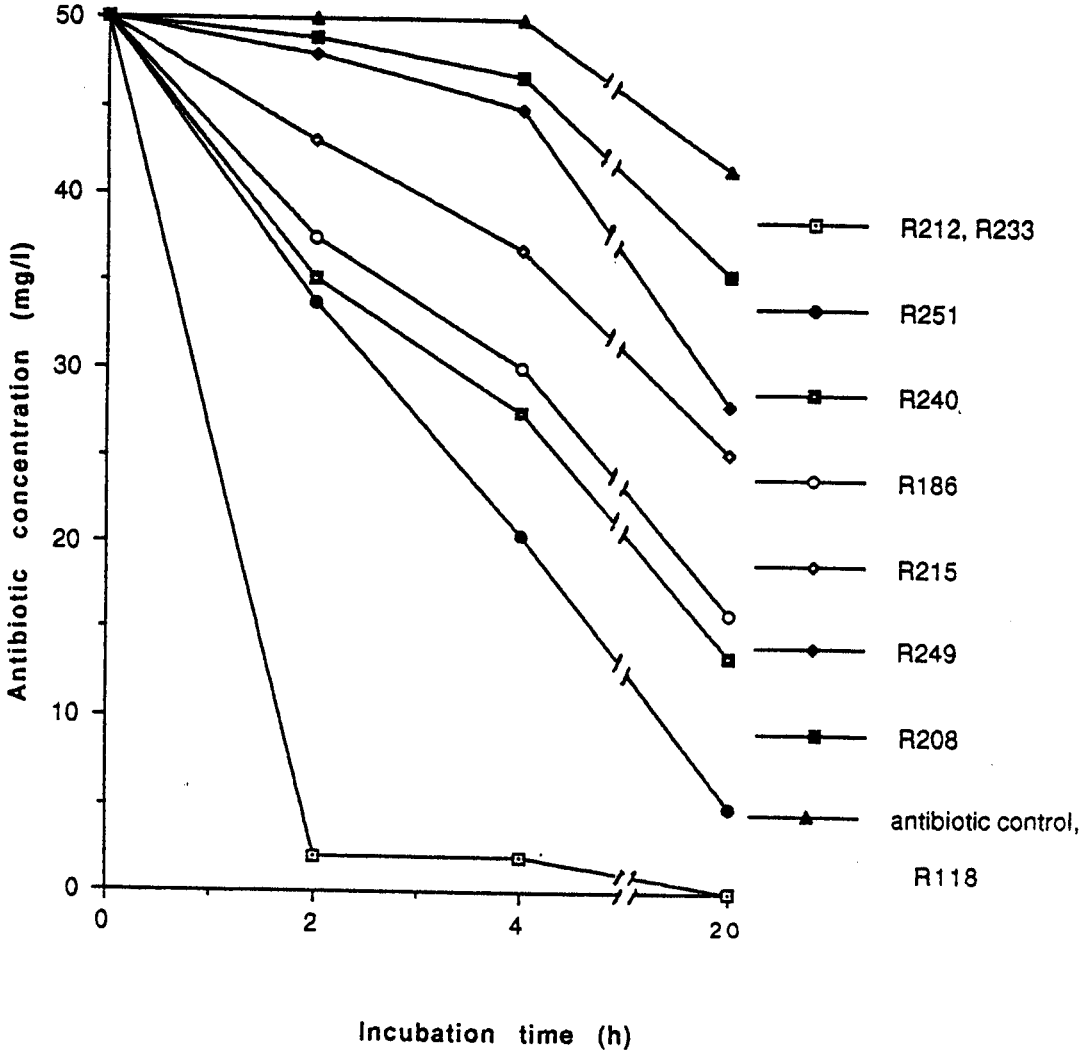


Fig. 4.3 Hydrolysis of latamoxef by enzyme extracts from bacteroides strains

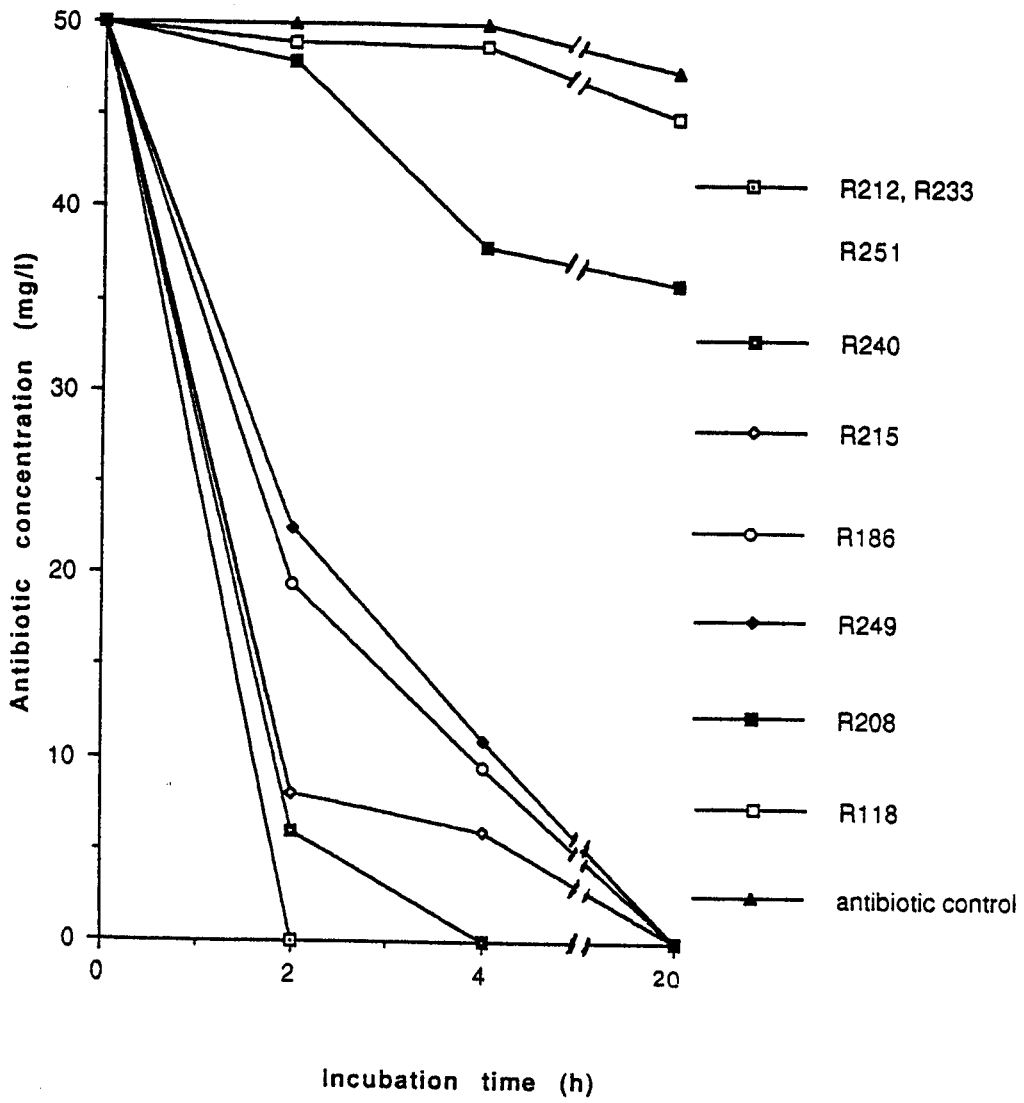
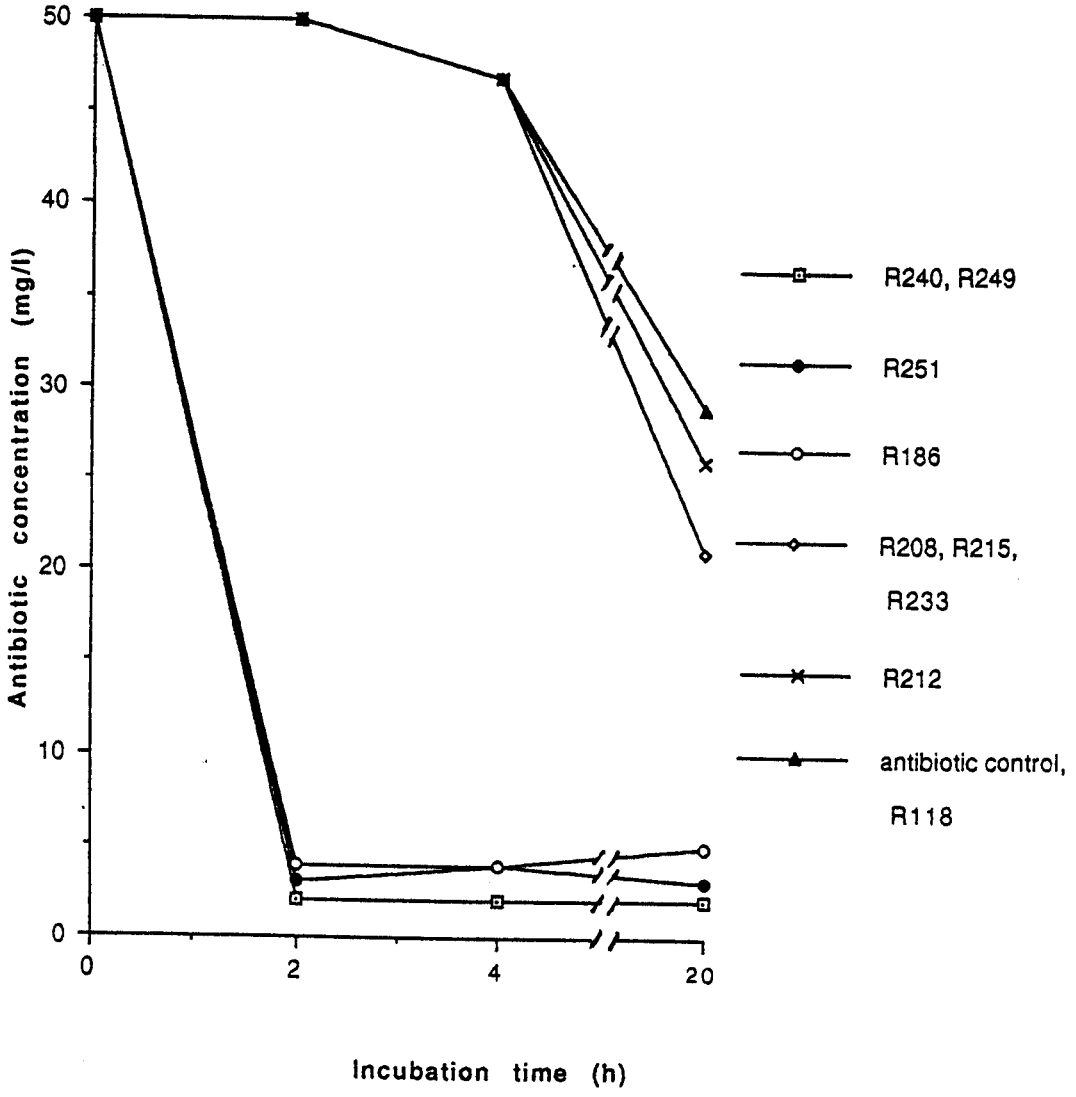


Fig. 4.4 Hydrolysis of imipenem by enzyme extracts from bacteroides strains



In the presence of clavulanic acid, benzylpenicillin was degraded by enzymes from all nine strains, although the enzymes from *B.fragilis* R208, *B.distasonis* R118 and *B.ovatus* R215 hydrolysed benzylpenicillin more slowly than those from the other strains. After 20 hours of incubation with extracts of *B.fragilis* R208 and *B.distasonis* R118, hydrolysis of benzylpenicillin was incomplete. Enzymes from *B.distasonis* R118 had little or no effect on cefoxitin or latamoxef in the test system. The other eight strains possessed enzymes capable of degrading cefoxitin and latamoxef, although only a minor degree of hydrolysis of these compounds by *B.fragilis* R208 was achieved. Latamoxef was hydrolysed more rapidly than cefoxitin in all cases. Only four of the isolates, *B.fragilis* R186, *B.fragilis* R240, *B.fragilis* R249 and *B.fragilis* R251, were active against imipenem. These isolates were able to hydrolyse the antibiotic within two hours, although a small amount of the compound was still detected after 20 hours of incubation. Interestingly, enzymes from all four of these strains were capable of complete hydrolysis of benzylpenicillin in the presence of clavulanic acid within 20 hours indicating a lack of protection by this inhibitor.

Concentrated crude cell extract from the control strain, *B.fragilis* NCTC 9344, had minimal effect on the antibiotics tested.

The atypical β-lactamases from bacteroides isolates which are able to hydrolyse β-lactamase stable compounds such as cefoxitin, latamoxef or imipenem are uncommon, comprising 6% of the strains in this study and 4% of strains examined in a similar study in 1986 (Eley and Greenwood, 1986a).

4:6 Hydrolysis of antibiotics by growing cultures of bacteroides which show reduced susceptibility

To investigate the possibility that the hydrolysis of β-lactam antibiotics by crude cell extracts did not reflect the behaviour of cell-bound enzyme, experiments were carried out with whole cells. The hydrolysis of sub-MIC concentrations of antibiotic in broth cultures by β-lactamases from growing cells was, therefore, assessed. The nine bacteroides isolates which produced elevated amounts of β-lactamase and showed reduced antibiotic susceptibility (See section 3.5) were examined. The following concentrations of antibiotic were added to the broth with the inoculum: benzylpenicillin 0.5 mg/l (in the presence of clavulanic acid 4 mg/l), cefoxitin 16 mg/l, latamoxef 16 mg/l and imipenem 0.5 mg/l. These antibiotic concentrations allowed growth of all the test strains except *B.fragilis* R186, R240 and R251, and *B.distasonis* R118 (with latamoxef), *B.ovatus* R215 (with benzylpenicillin), and *B.ovatus* R102 (with imipenem). The antibiotic residues, measured by microbiological assay, present after 22 hours incubation are shown as percentages of the antibiotic control (organism free) in Table 4.4.

Benzylpenicillin, in the presence of clavulanic acid, was markedly degraded only by *B.fragilis* R186, R212, R233, R240 and R251. A residue of cefoxitin of >50% of the control value was found in all cultures except *B.fragilis* R212, *B.ovatus* R215 and *B.thetaiotaomicron* R233. No latamoxef (<10% of control) was detected after 22 hours incubation with *B.fragilis* R212 and *B.ovatus* R215, and >60% of the control value remained with *B.ovatus* R102, *B.fragilis* R208 and *B.thetaiotaomicron* R233. More than 50% of the control concentration of imipenem was detected after the incubation

Table 4.4 Antibiotic remaining after 22 hours of incubation with resistant isolates of bacteroides as a percentage of the organism free control, measured by microbiological assay.

Bacterial strain	Percentage of antibiotic remaining			
	imipenem (0.5 mg/l)	latamoxef (16 mg/l)	cefoxitin (16 mg/l)	benzyl-penicillin* (0.5 mg/l)
B.fragilis R186	<30+	NG	64	<15+
B.fragilis R208	66	70	73	51
B.fragilis R212	75	<10+	<20+	<15
B.fragilis R240	<30	NG	55	<15
B.fragilis R251	<30	NG	60	<15
B.distasonis R118	55	NG	70	55
B.ovatus R102	NG	81	88	88
B.ovatus R215	52	<10	48	NG
B.thetaiotaomicron R233	57	62	<20	<15

NG = no growth

* In the presence of clavulanic acid 4 mg/l

\+ Limit of detection

Figures in brackets indicate the initial concentration of antibiotic

period in cultures of *B.fragilis* R208 and R212, *B.distasonis* R118, *B.ovatus* R215 and *B.thetaiotaomicron* R233. No imipenem (<30% of the antibiotic control) was detected in the cultures of *B.fragilis* R186, R240 and R251.

Inactivation of β-lactamase stable β-lactam antibiotics by potent β-lactamases produced by whole cells of *Bacteroides* species have been previously reported (Cuchural *et al.*, 1983; Yotsuji *et al.*, 1983; Eley and Greenwood, 1986b). This study showed generally good correlation between the degree of hydrolysis of β-lactams due to concentrated crude β-lactamase extracts and whole growing cells. Two exceptions, however, were apparent. *B.thetaiotaomicron* R233 displayed complete breakdown of latamoxef with the crude enzyme extract but less than 50% inactivation of the antibiotic with whole cells. This may have been due to the concentrated state of the β-lactamases in the crude extract. Also, *B.distasonis* R118 caused some breakdown of cefoxitin and imipenem with whole cells only. It is possible that inactivation of the β-lactamases of this strain occurred during the sonication process of the crude cell extract preparation, although this extract was able to partially breakdown benzylpenicillin.

4:7 Classification

The 21 β-lactamases were grouped into four distinct types, as summarised in Table 4.5.

The first group consisted of the enzymes produced by *B.fragilis* R186, R240, R249 and R251. These had broadly similar characteristics: low specific cephalosporinase activity; insusceptibiliy to inhibition by β-lactamase inhibitors, including clavulanic acid; relative resistance to imipenem. A distinctive feature of these strains was the ability of crude enzyme extracts and whole cells to degrade

Table 4.5 Characteristics of four types of β-lactamases from 24 bacteroides strains

Type	MIC (mg/l) of benzyl penicillin	cefoxitin	latamoxef	imipenem	Inhibitors of β-lactamase
1	4-16	16-32	0.5-4	1-4	Resistant
2	>16	>32	>128	1-4	Intermediate
3	>16	32	>8	2-4	Variable
4	4->64	4-32	0.5-32	≤1	Susceptible

Type	Hydrolysis of benzyl penicillin*	cefoxitin	latamoxef	imipenem	Activity of benzylpenicillin restored by clavulanic acid
1	Yes	Slow	Yes	Yes	No
2	Yes	Yes	Yes	No	Partial
3	Slow	No	No	No	Yes
4	No	No	No	No	Yes

* In the presence of clavulanic acid

imipenem efficiently. These enzymes also hydrolysed latamoxef and, more slowly, cefoxitin.

The second group was represented by *B.fragilis* R212, *B.ovatus* R215 and *B.thetaiotaomicron* R233. These strains exhibited resistance to benzylpenicillin, cefoxitin and latamoxef, and reduced susceptibility to imipenem. The β-lactamases displayed intermediate or high specific activity and showed similar but not identical inhibition profiles. These enzymes hydrolysed β-lactamase-stable compounds other than imipenem, although the breakdown of cefoxitin by *B.ovatus* R215 was modest, as was the degradation of latamoxef by whole cells of *B.thetaiotaomicron* R233. These β-lactamases were not fully susceptible to clavulanic acid, and the inhibitor failed to offer a high degree of protection to benzylpenicillin in hydrolysis studies and, in the case of *B.fragilis* R212 and *B.thetaiotaomicron* R233, MIC titrations. Their isoelectric points were diverse, as would be expected from these different species, indicating that the enzymes are not identical.

The third group was composed of two strains, *B.distasonis* R118 and *B.fragilis* R208, which exhibited reduced susceptibility to cefoxitin and imipenem, and, in the case of *B.fragilis* R208, latamoxef. However, these antibiotics were hydrolysed poorly, or not at all, by the crude enzyme extracts and whole growing cells. The enzymes displayed variable inhibition profiles and intermediate levels of specific activity. The activity of benzylpenicillin with both strains was restored in the presence of clavulanic acid, although these enzymes were not fully susceptible to the inhibitor in inhibition studies and slow hydrolysis of benzylpenicillin was observed in the presence of clavulanic acid.

Most of the strains encountered in this study retained a high

degree of susceptibility to cefoxitin, latamoxef and imipenem, and belong to group four. Generally, these enzymes were fully susceptible to β-lactamase inhibitors, and clavulanic acid fully restored the activity of benzylpenicillin. These strains produced enzymes with a wide range of specific activities that had no hydrolytic activity against β-lactamase-stable compounds. Their isoelectric points were all between 4.5 and 5.3.

4:8 Discussion

The typical β-lactamase produced by *B.fragilis* has been well characterised, and categorised as a member of group 2e of the Bush classification scheme (Bush, 1989c). There is also a wide variety of β-lactamases, from *B.fragilis* and other *Bacteroides* species, which are more difficult to classify. Some have been allocated a position in the Bush classification scheme in groups 1, 2d, 3 and 4 (Bush 1989b,c).

A general classification scheme for the diverse bacteroides β-lactamases would be beneficial. Two schemes have been put forward by Eley and Greenwood (1986a,b). Using the characteristics of type culture strains and bacteroides known to hydrolyse cefoxitin, latamoxef or imipenem, they defined three broad groups on the basis of antibiotic hydrolysis and inhibitor profiles. The other study examined clinical isolates of bacteroides and reported three groups based on specific activity together with these other characteristics.

In this study, four enzyme types are apparent from the characteristics of the sensitivity and hydrolysis of benzylpenicillin and β-lactamase stable β-lactam antibiotics, inhibitor profiles and effect of clavulanic acid on the activity of benzylpenicillin, together with

specific activity and isoelectric point.

These types appear to have features similar to those of bacteroides β-lactamases described by other workers. The properties of strains of group one are similar to those previously described for *B.fragilis* 57, 97 and 119 by Eley and Greenwood (1989a,b) in that they inactivated cefoxitin, latamoxef and imipenem and were insusceptible to β-lactamase inhibitors. Type one strains appear to be similar to those belonging to Bush's class three which produce carbapenem hydrolysing metallo-β-lactamases (Cuchural *et al.*, 1986b; Bush, 1989c; Bandoh *et al.*, 1991; Ajiki *et al.*, 1991; Hedberg *et al.*, 1992). The failure to detect isoelectric focusing bands in three of the four *B.fragilis* strains of this group (R240, R249 and R251) is anomalous and may reflect the low cephalosporinase activity of these strains resulting in poor breakdown of nitrocefin, and hence poor visibility, of the bands which represent the position of β-lactamase on the isoelectric focusing membrane. Interestingly, the two *B.fragilis* strains (TAL 2480 and 3636) reported by Cuchural *et al.* (1986b), which produced zinc dependent carbapenemases, failed to focus well enough to establish reliable pI values.

The β-lactamase types of group two are similar to those enzymes produced by *B.fragilis* 0423, *B.thetaiotaomicron* 0456 and *B.distasonis* R939 as previously described by Eley and Greenwood (1986b). These strains are resistant to, and inactivate, latamoxef and cefoxitin. They are more resistant than the type-culture strain to imipenem, but do not inactivate it. Strain *B.fragilis* 107, described by Eley and Greenwood (1986a), has features similar to those of group two except that it is generally more sensitive. This group of β-lactamases also appears similar to other enzymes reported in the literature, such as those from *B.distasonis* TAL 7860 and

B.uniformis 2986, which hydrolysed cefoxitin and showed at least partial inhibition with clavulanic acid (Olsson-Liljequist *et al.*, 1980; Hurlbut *et al.*, 1990). The isoelectric points of these enzymes do, however, cover a wide range.

Type three is difficult to relate to other unusual β-lactamases produced by bacteroides which have been described by other workers and categorised in the Bush classification scheme as belonging to groups 1, 2d and 4. (Sato *et al.*, 1982; Yotsuji *et al.*, 1983; Bush, 1989b,c). In many ways they resemble the type four enzymes of this present study, and may be related to this group, except for increased resistance to β-lactamase stable β-lactam antibiotics and generally reduced susceptibility to β-lactamase inhibitors.

Enzymes of type four appear to represent the typical cephalosporinase that most bacterodes are known to produce and belong to group 2e of the Bush classification scheme (Bush, 1989c). The strains tested were chosen because of their ability to produce enhanced levels of β-lactamase by the nitrocefin screening test. Type four strains, therefore, probably produce greater than normal amounts of this common enzyme: *B.fragilis* 2013E described by Eley and Greenwood (1986b) as a high producer of typical *B.fragilis* β-lactamase is likely to belong to this group.

Groups one, two and four in this study, therefore, appear consistent with the three categories described by Eley and Greenwood (1986b).

Strains within groups two and three (*B.fragilis* R208 and R212, *B.distasonis* R118, *B.ovatus* R215 and *B.thetaiotaomicron* R233) and *B.ovatus* R102 have been described which have reduced susceptibility to cefoxitin, latamoxef or imipenem and possess β-

lactamases incapable of marked hydrolysis of these compounds. This suggests that increased resistance in these strains is due mechanisms other than β-lactamase hydrolysis.

The remainder of this study was devoted to examining resistance of *B.fragilis* to the carbapenems. Strains of groups one, two and three contain bacteroides isolates with reduced susceptibility to imipenem, having MICs of >1 mg/l compared to 'normal' sensitive strains with MICs of 0.06 mg/l - 0.12 mg/l. Those of group one, *B.fragilis* R186, R240 and R251, produced β-lactamases that hydrolyse imipenem rapidly. Increased resistance of these strains, therefore, appears to be associated with carbapenemases and this was examined further. Strains of types two and three, including *B.fragilis* R208 and R212, are incapable of rapid hydrolysis of imipenem, suggesting other mechanisms of resistance, which were also the subject of further examination.

CHAPTER 5

CARBAPENEMASE ACTIVITY ASSOCIATED WITH *B.FRAGILIS* SHOWING INCREASED RESISTANCE TO CARBAPENEMS

5:1 Introduction

5:2 Susceptibility and imipenem stability of additional *B.fragilis* strains

5:3 Hydrolysis of imipenem by *B.fragilis* strains exhibiting reduced susceptibility to the drug

(1) Hydrolysis of imipenem 0.5 mg/l by growing *B.fragilis* cultures

(2) Hydrolysis of imipenem 50 mg/l by whole cells of *B.fragilis*

5:4 Turbidimetry and inoculum effect

5:5 Effect of clavulanic acid on broth dilution MICs of imipenem

5:6 Comparative susceptibility and stability of imipenem and meropenem

5:7 Specific imipenemase activity

5:8 Detection of metallo-β-lactamases

(1) with imipenem as substrate

(2) with nitrocefin as substrate

5:9 Effect of zinc acetate on the imipenem susceptibility of carbapenemase producing strains

5.10 Discussion

CHAPTER 5

CARBAPENEMASE ACTIVITY ASSOCIATED WITH *B.FRAGILIS* SHOWING INCREASED RESISTANCE TO CARBAPENEMS

5:1 Introduction

Of 108 clinical isolates of bacteroides, seven showed increased resistance to imipenem (MIC >1 mg/l). All seven isolates belonged to the group of 21 strains possessing elevated levels of β-lactamase as judged by the semi-quantitative whole cell test with nitrocefin. Five of these seven strains were *B.fragilis* (R186, R208, R212, R240, R251) which were studied in detail to elucidate the mechanisms of resistance to imipenem. In addition, *B.fragilis* 2013E, 0423 and 119, from the departmental culture collection, were examined. These three strains have previously been reported as having increased resistance to imipenem by Eley and Greenwood (1986b) and represent the different *B.fragilis* types described in section 4:8. Previous work in this study (Chapter 4) and by Eley and Greenwood (1986b) indicated that *B.fragilis* R186, R240 R251 and 119 produced β-lactamases capable of hydrolysis of imipenem.

The production, activity and characteristics of carbapenemases from the resistant *B.fragilis* strains were examined together with the role of these enzymes in carbapenem resistance. Hydrolysis of imipenem by whole cells of these eight isolates was again examined to determine the rate of antibiotic degradation, and the residual imipenem concentrations were measured by two assay methods and compared. The impact of dense inocula on the activity of imipenem

against these strains was investigated, as was the presence of the β-lactamase inhibitor, clavulanic acid. The comparative susceptibility of imipenem and meropenem to these carbapenemases was determined. The specific imipenemase activites of the enzymes were calculated and correlated with imipenem resistance. Also, the carbapenemases were characterised in terms of cation dependency.

5:2 Susceptibility and imipenem stability of additional *B.fragilis* strains

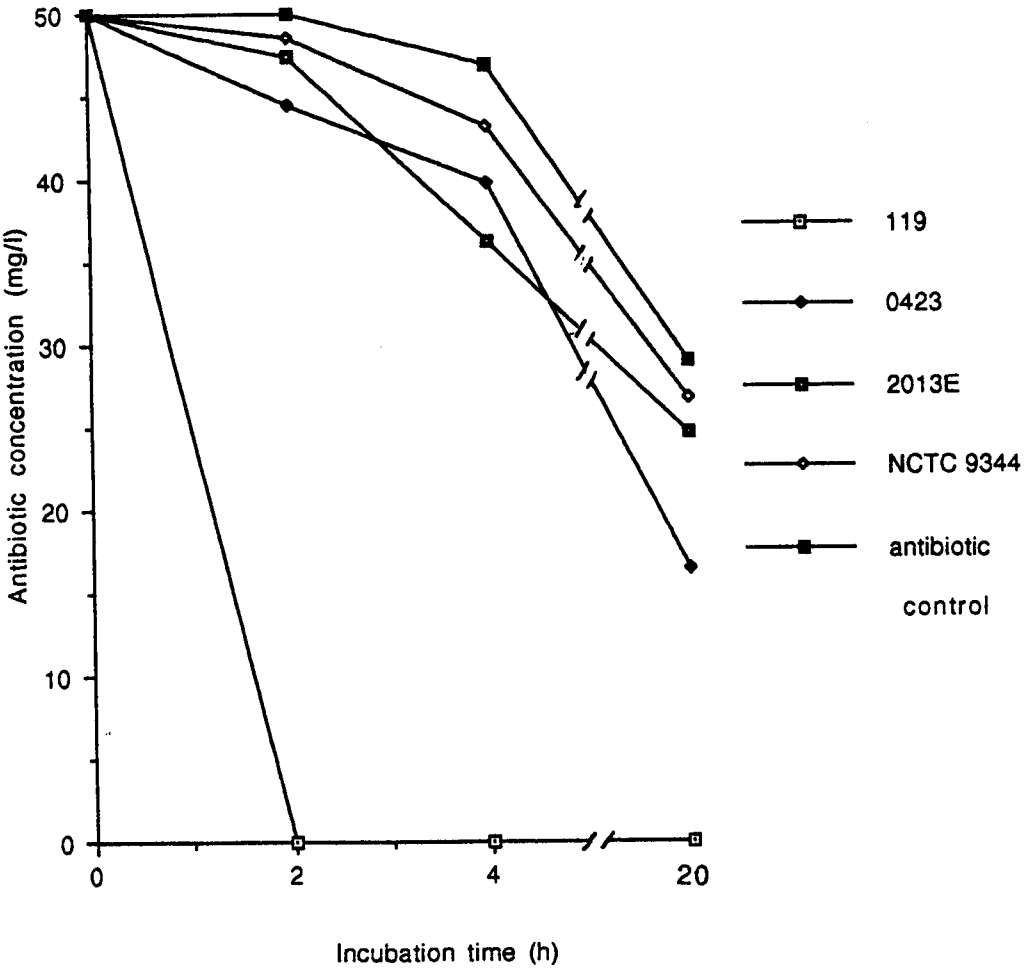
The MICs of imipenem obtained by the agar incorporation method (BHIS agar) after 48 hours of incubation were found to be 2 mg/l for *B.fragilis* 2013E and 0423, and 16 mg/l for *B.fragilis* 119. These results were identical to, or within one dilution of, those values obtained by Eley and Greenwood (1986b).

Rapid hydrolysis of imipenem by concentrated crude cell extracts was shown only with *B.fragilis* 119, which totally degraded imipenem 50 mg/l within two hours of incubation (Fig. 5.1). The enzymes from *B.fragilis* 0423 and 2013E caused slow hydrolysis of imipenem so that after 20 hours of incubation 55% and 83% of the control remained respectively. The amount of imipenem present was only minimally reduced by enzymes from control strain NCTC 9344. Eley and Greenwood (1986b) also reported complete hydrolysis of imipenem by *B.fragilis* 119, but they observed no detectable breakdown of imipenem with *B.fragilis* 0423 and 2013E.

5:3 Hydrolysis of imipenem by *B.fragilis* strains exhibiting reduced susceptibility to the drug

The hydrolysis of imipenem was assessed at two concentrations, 0.5 mg/l and 50 mg/l, with whole cells of *B.fragilis*

Fig. 5.1 Hydrolysis of imipenem by enzyme extracts of *B.fragilis* culture collection strains with increased resistance to the drug



strains showing increased resistance to imipenem. Tests were carried out in BHIS broth.

The lower imipenem concentration, 0.5 mg/l, which was below the MIC for the moderately resistant test strains, was added with the inoculum at the begining of the experiment. The speed of hydrolysis by β-lactamases from these growing cells was determined by measuring the concentration of imipenem by microbiological assay after 6 and 22 hours incubation.

The higher imipenem concentration, 50 mg/l, was also used to assess hydrolysis by enzymes produced by whole cells and to compare the measurements of residual imipenem as determined by microbiological assay and HPLC. In these experiments, antibiotic was added to bacterial cultures in the late logarithmic phase of growth. Growth was monitored turbidimetrically and drug added when growth had raised the opacity to a level of 70% of maximum, equivalent to a viable count of *ca* 10^8 cfu/ml.

(1) Hydrolysis of imipenem 0.5 mg/l by growing *B.fragilis* cultures

With *B.fragilis* R186, R240, R251 and 119 between 29% and 44% of the control concentration of imipenem was lost after 6 hours of incubation and <30% of the organism-free control value remained after 22 hours (Table 5.1). Cultures of *B.fragilis* R208, R212, 2013E and 0423 had little or no effect on the imipenem concentration after 6 hours of incubation and between 54% and 75% of the control value remained after 22 hours.

The rate of hydrolysis of imipenem by whole cells appeared to be less than that with concentrated crude cell extracts. After two hours exposure with crude extract of type one enzymes, hydrolysis of imipenem 50 mg/l was virtually complete. Using growing cells and

Table 5.1 Hydrolysis of imipenem (0.5 mg/l) by cultures of *B.fragilis* that exhibited reduced susceptibility to the drug.

Bacterial strain		Percentage of imipenem control remaining* after 6h	22h
B.fragilis	R186	68	<30+
B.fragilis	R240	56	<30
B.fragilis	R251	71	<30
B.fragilis	119	60	<30
B.fragilis	R208	100	54
B.fragilis	R212	100	60
B.fragilis	2013E	100	75
B.fragilis	0423	93	62

+ limit of detection = 0.06 mg/l

* Measured by microbiological assay with a clinical isolate of *Strep. pyogenes* as indicator organism.

one hundreth of that imipenem concentration, more than 50% of the imipenem remained after six hours. After 20 hours incubation, with both the whole cells (Table 5.1) and concentrated cell extract (Figs. 4.4 and 5.1), type one enzymes (*B.fragilis* R186, 240, 251 and 119) hydrolysed imipenem to <30% of the antibiotic control, and with types two and three (*B.fragilis* R208, R212, 2013E 0423) >50% of the antibiotic remained.

(2) Hydrolysis of imipenem 50 mg/l by whole cells of *B.fragilis*

Both microbiological assay and HPLC revealed that ≥60% of the imipenem control concentration remained after 18 hours of incubation in the presence of whole cells of *B.fragilis* R208, R212 2013E and 0423 (Table 5.2). *B.fragilis* R186, R240 and 119, however, caused a marked hydrolysis of imipenem with no drug detected after 18 hours of incubation. *B.fragilis* R251 also caused a substantial reduction in the residual imipenem concentration with <20% of the control remaining.

The results obtained with the two assay methods were generally similar, although those obtained by HPLC tended to be higher compared to imipenem concentrations measured by microbiological assay. Hydrolysis of imipenem (50 mg/l) by crude cell extracts measured by HPLC after 20 hours of incubation (Fig. 4:4) resulted in imipenem residues similar to those seen with whole cells (Table 5.2).

5:4 Turbidimetry and inoculum effect

The effect of imipenem on inocula of *ca.* 10^5 and 10^8 cfu/ml was examined turbidimetrically to investigate the influence of β-

Table 5.2 Hydrolysis of imipenem (50 mg/l) by late-logarithmic phase cultures of *B.fragilis*. Results expressed as percentage of antibiotic control remaining after incubation for 18 hours, as judged by biological assay and HPLC.

Bacterial strain	Percentage imipenem remaining: Biological assay*	HPLC#	
B.fragilis R186	<5+	<15++	(17)
B.fragilis R240	<5	<15	(10)
B.fragilis R251	11	19	(14)
B.fragilis 119	<5	<15	(<5)
B.fragilis R208	84	90	(72)
B.fragilis R212	100	100	(90)
B.fragilis 2013E	60	88	(83)
B.fragilis 0423	65	70	(55)
B.fragilis NCTC 9344	100	100	(93)

* with 'Oxford' Staphylococcus as indicator organism and residue diluted 1 in 10 before assay

\+ Limit of detection (=0.12 mg/l)

++ Limit of detection (=5 mg/l)

In parentheses: results after exposure of imipenem (50 mg/l) to crude cell extracts for 20 hours of incubation measured by HPLC (from Figs 4.4 and 5.1)

lactamase on the response of whole cells to the antibiotic. In this system, susceptibility of the antibiotic to a particular β-lactamase should be reflected in an altered response of the larger inoculum. Greater numbers of bacteria should result in an increase in the total amount of intracellular β-lactamase present in the culture which is available to inactivate the susceptible β-lactam antibiotic, leading to an increase in resistance.

Continuous opacity records obtained in low and high inoculum experiments with each of the *B.fragilis* strains are given in Figs. 5.2-5.19. A summary of the results of these turbidimetric data in terms of MACs (lowest antibiotic concentration causing deviation from normal growth) and MICs (lowest antibiotic concentration required to suppress growth for the whole period of observation) for each inoculum is given in Table 5.3.

An inoculum effect that was reflected most markedly in a change in the MAC of imipenem, but also to a lesser extent in the turbidimetric MIC, was observed with those strains that produced enzymes able to degrade imipenem, i.e. *B.fragilis* R186, R240, R251 and was particularly marked with *B.fragilis* 119. For these strains, MACs of imipenem in the high inoculum experiments were at least eight fold higher than those obtained with the low inoculum, and the MICs for the high inoculum were at least twice the low inoculum MICs. No inoculum effect was observed with *B.fragilis* R212 and 0423. *B.fragilis* R208 and 2013E showed a slight inoculum effect, the MACs of imipenem increasing by a factor of two as the inoculum was raised. This was also the case for the *B.fragilis* NCTC 9344 control. These results indicate that imipenem is susceptible to β-lactamases of *B.fragilis* R186, R240, R251 and 119, and these β-lactamases are a factor in the resistance of these strains to the drug.

Fig. 5.2 Continuous opacity records of *B.fragilis* R186 exposed at time zero to imipenem at the concentrations shown (mg/l). The inoculum was *ca.* 10^5 cfu/ml in each case

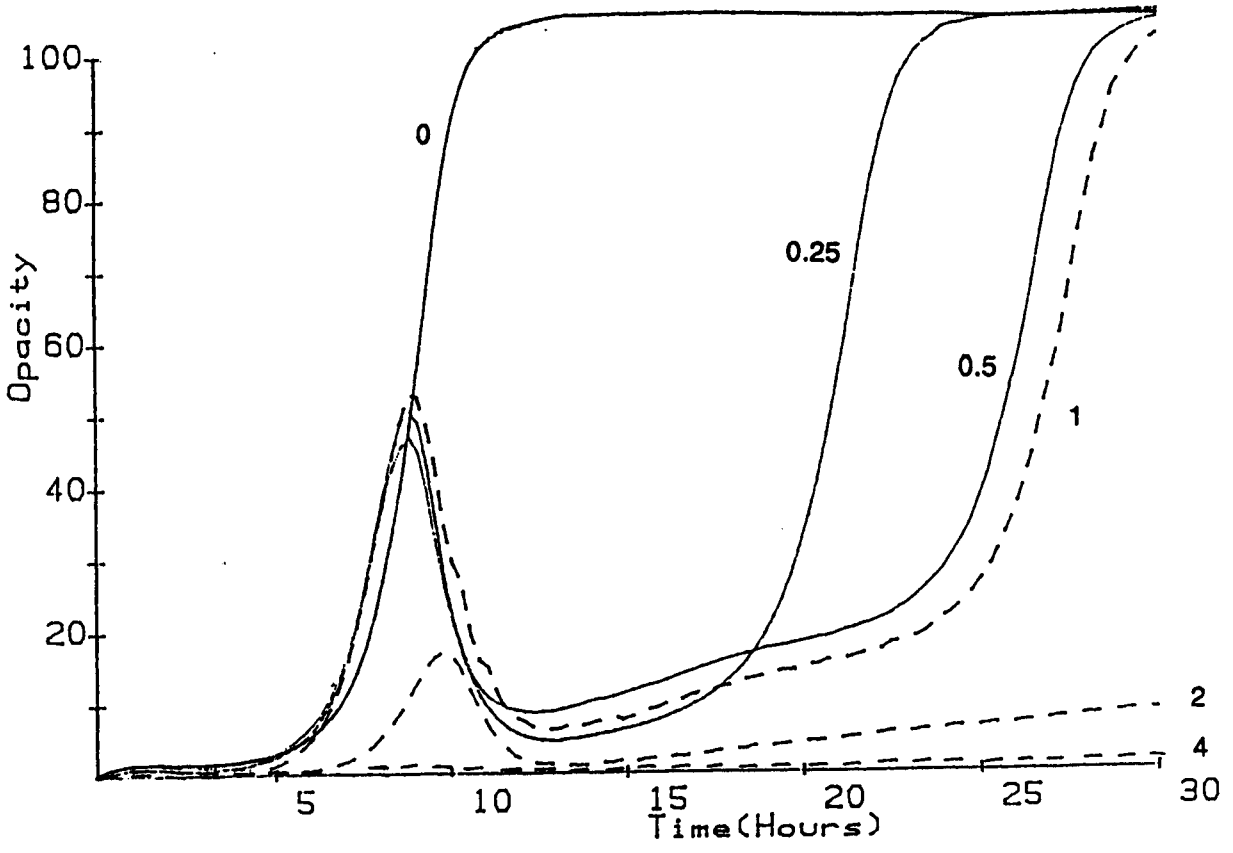


Fig. 5.3 Continuous opacity records of *B.fragilis* R186 with imipenem added at 30% opacity (indicated by arrow) to achieve the concentrations (mg/l) shown

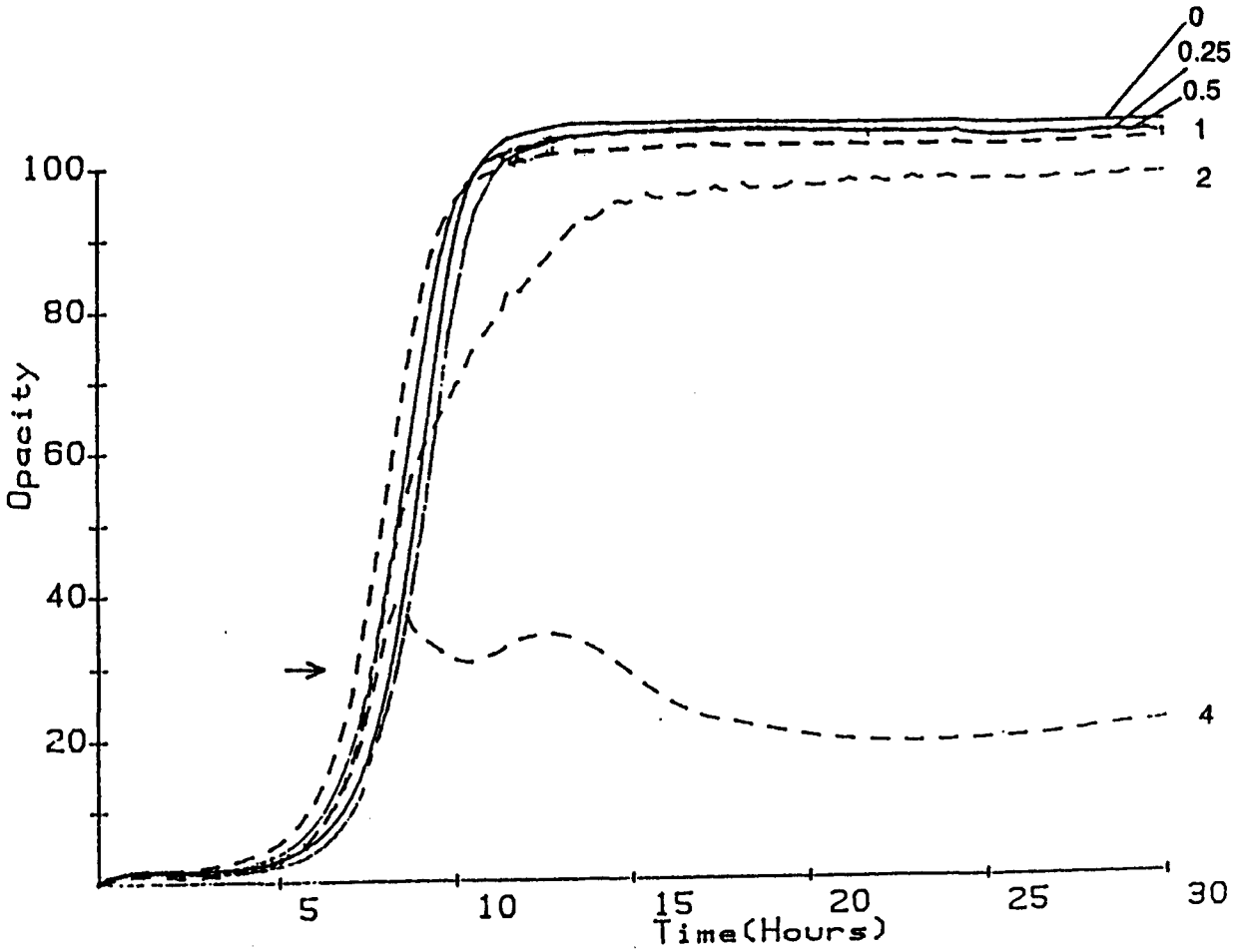


Fig. 5.4 Continuous opacity records of *B.fragilis* R240 exposed at time zero to imipenem at the concentrations shown (mg/l). The inoculum was *ca.* 10^5 cfu/ml in each case

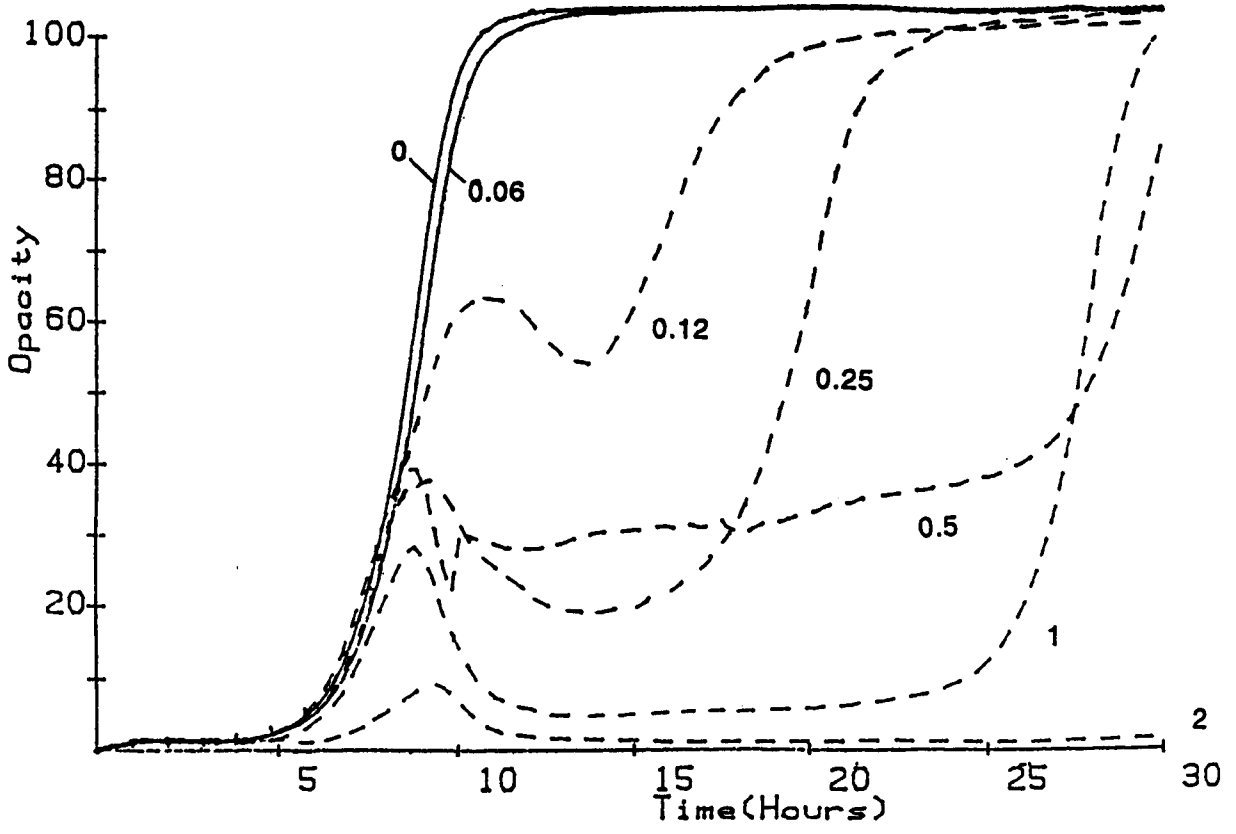


Fig. 5.5 Continuous opacity records of *B.fragilis* R240 with imipenem added at 30% opacity (indicated by arrow) to achieve the concentrations (mg/l) shown

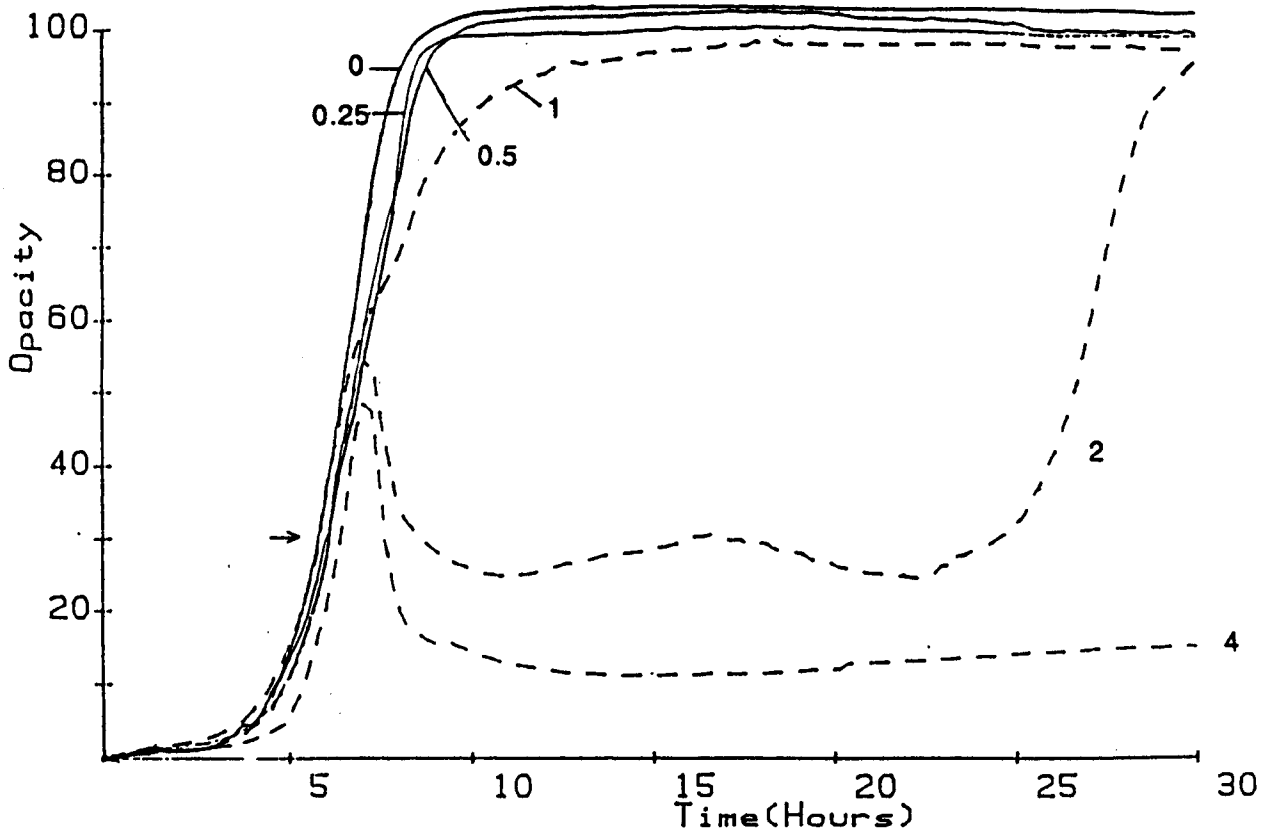


Fig. 5.6 Continuous opacity records of *B.fragilis* R251 exposed at time zero to imipenem at the concentrations shown (mg/l). The inoculum was *ca.* 10^5 cfu/ml in each case

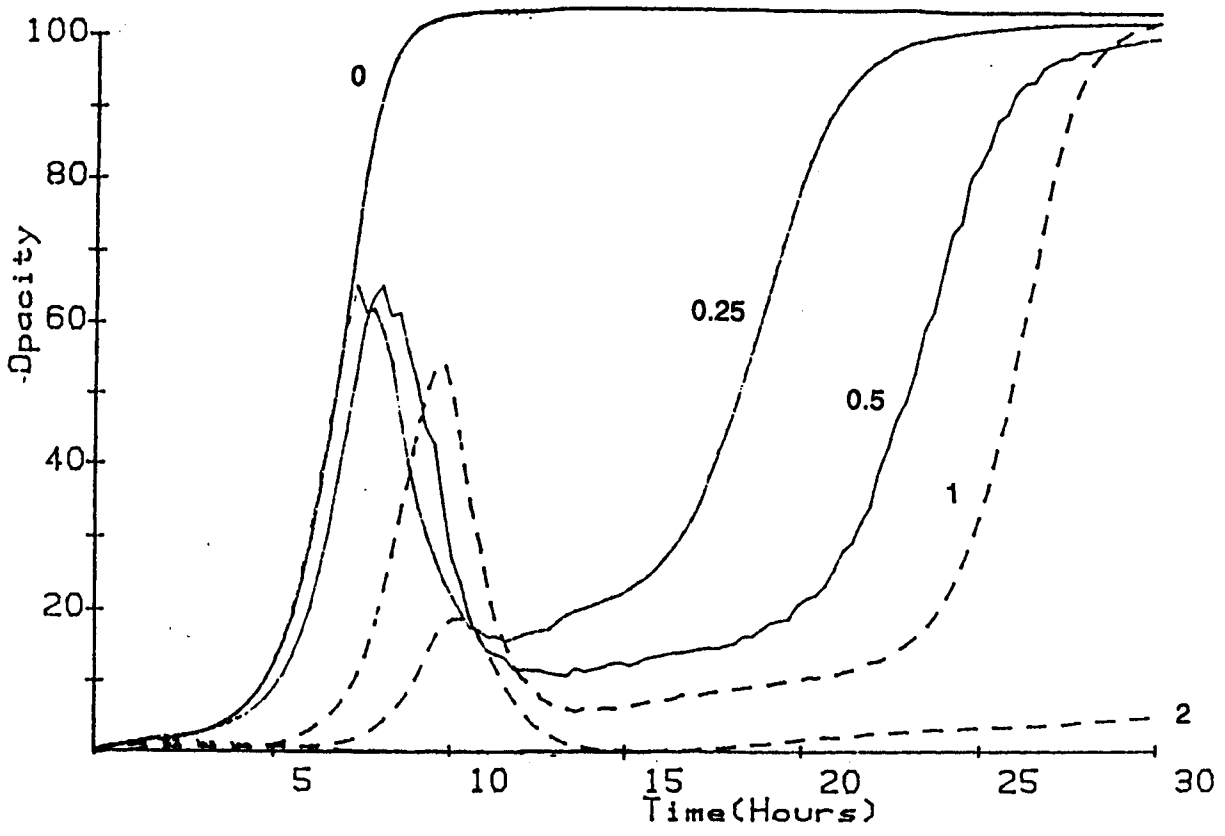


Fig. 5.7 Continuous opacity records of *B.fragilis* R251 with imipenem added at 30% opacity (indicated by arrow) to achieve the concentrations (mg/l) shown

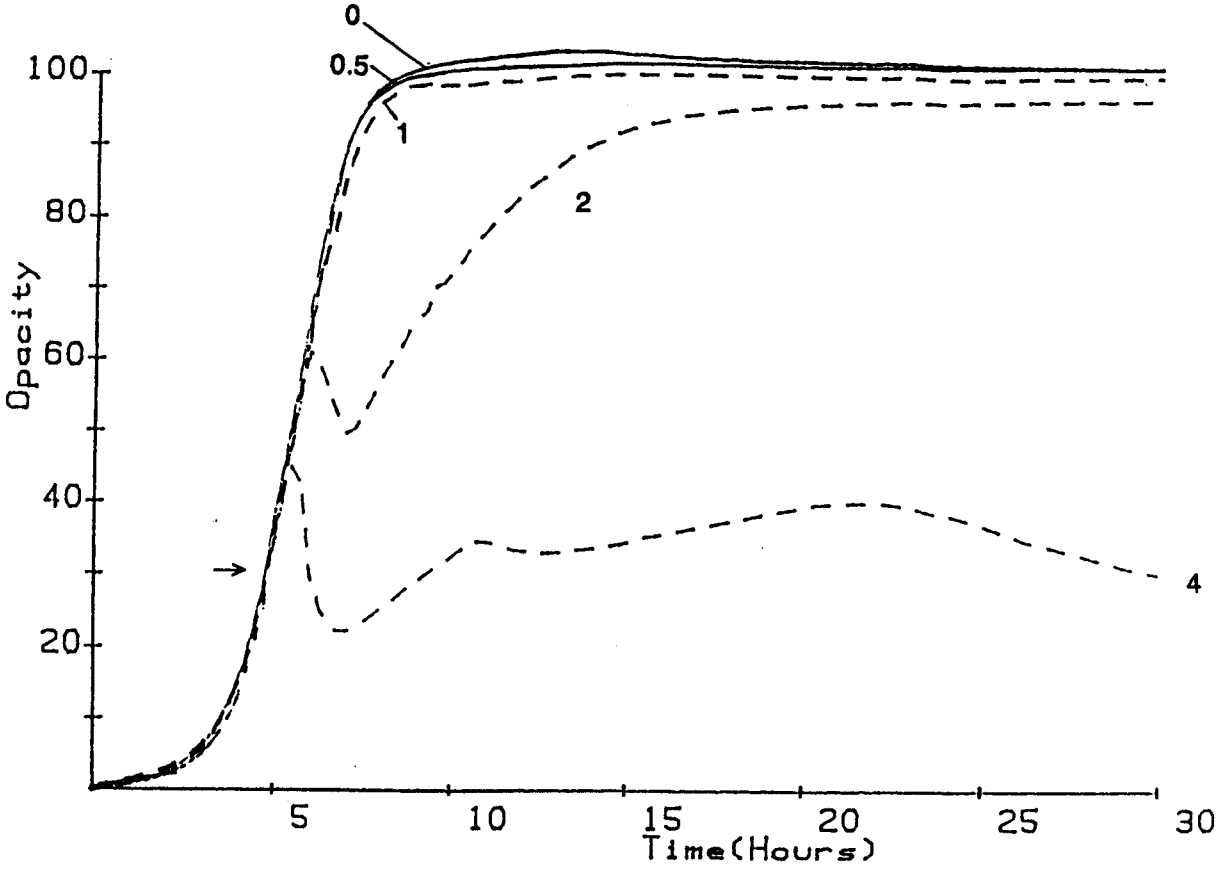


Fig. 5.8 Continuous opacity records of *B.fragilis* 119 exposed at time zero to imipenem at the concentrations shown (mg/l). The inoculum was *ca.* 10^5 cfu/ml in each case

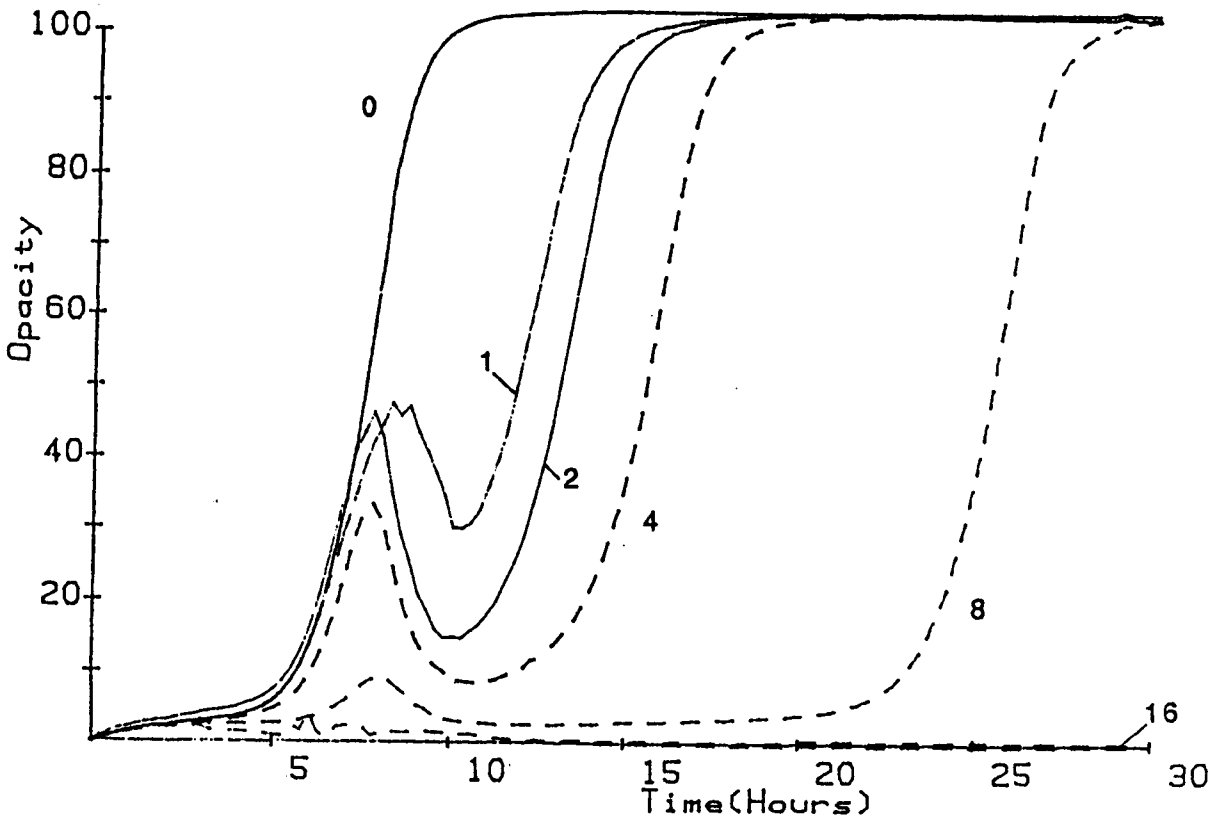


Fig. 5.9 Continuous opacity records of *B.fragilis* 119 with imipenem added at 30% opacity (indicated by arrow) to achieve the concentrations (mg/l) shown

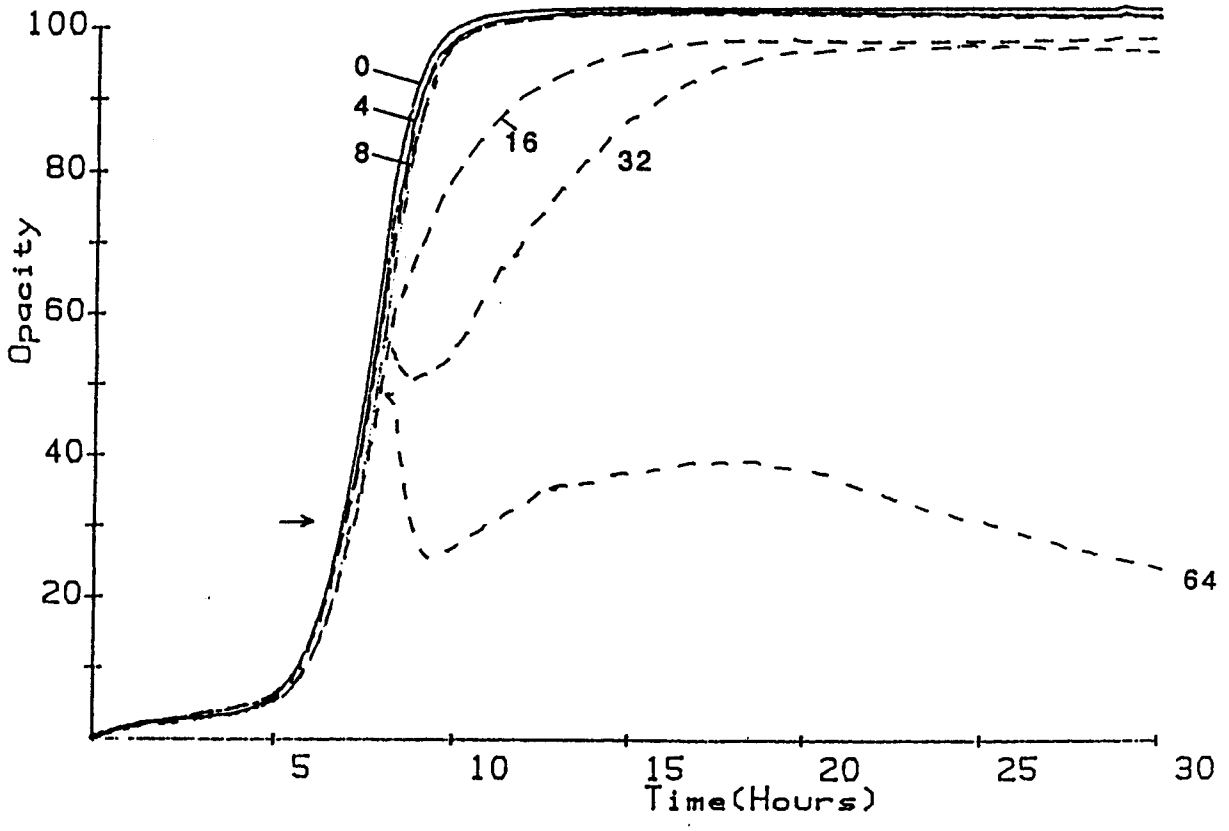


Fig. 5.10 Continuous opacity records of *B.fragilis* R208 exposed at time zero to imipenem at the concentration shown (mg/l). The inoculum was *ca.* 10^5 cfu/ml in each case.

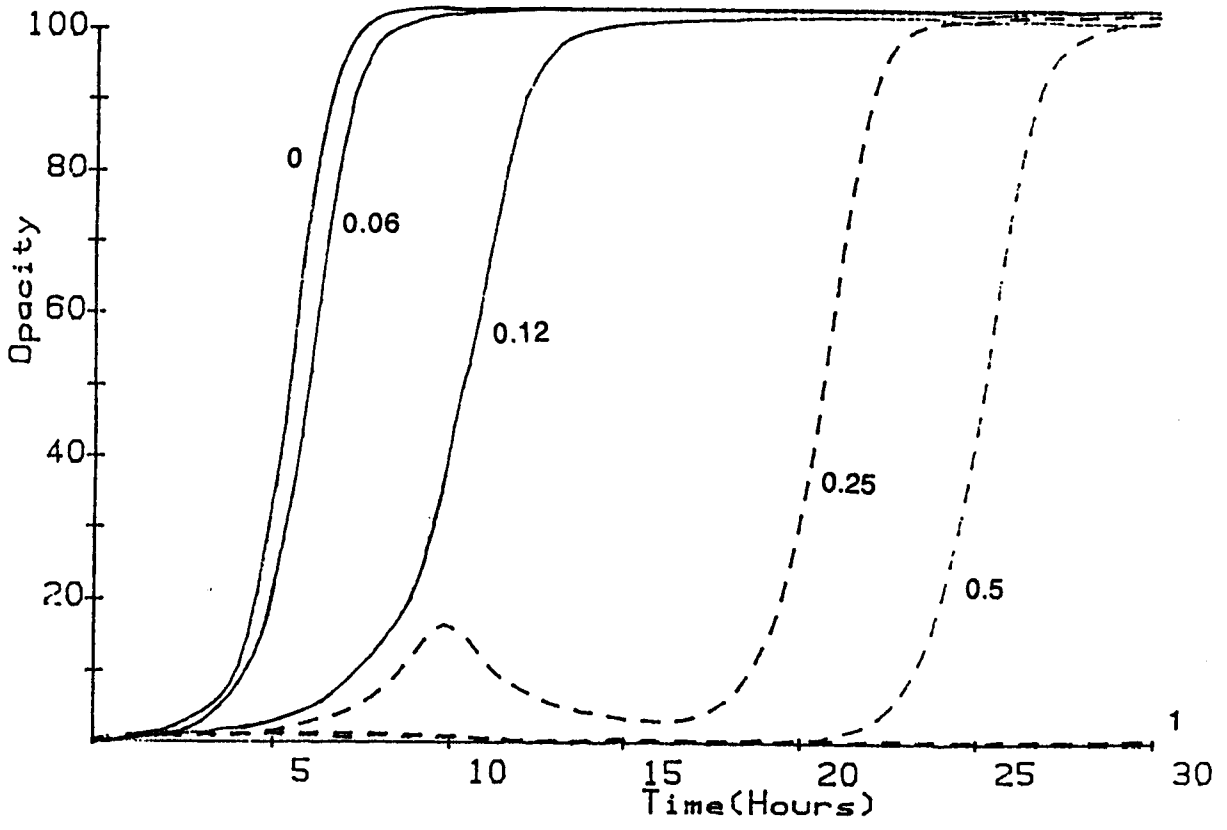


Fig. 5.11 Continuous opacity records of *B.fragilis* R208 with imipenem added at 30% opacity (indicated by arrow) to achieve the concentration (mg/l) shown

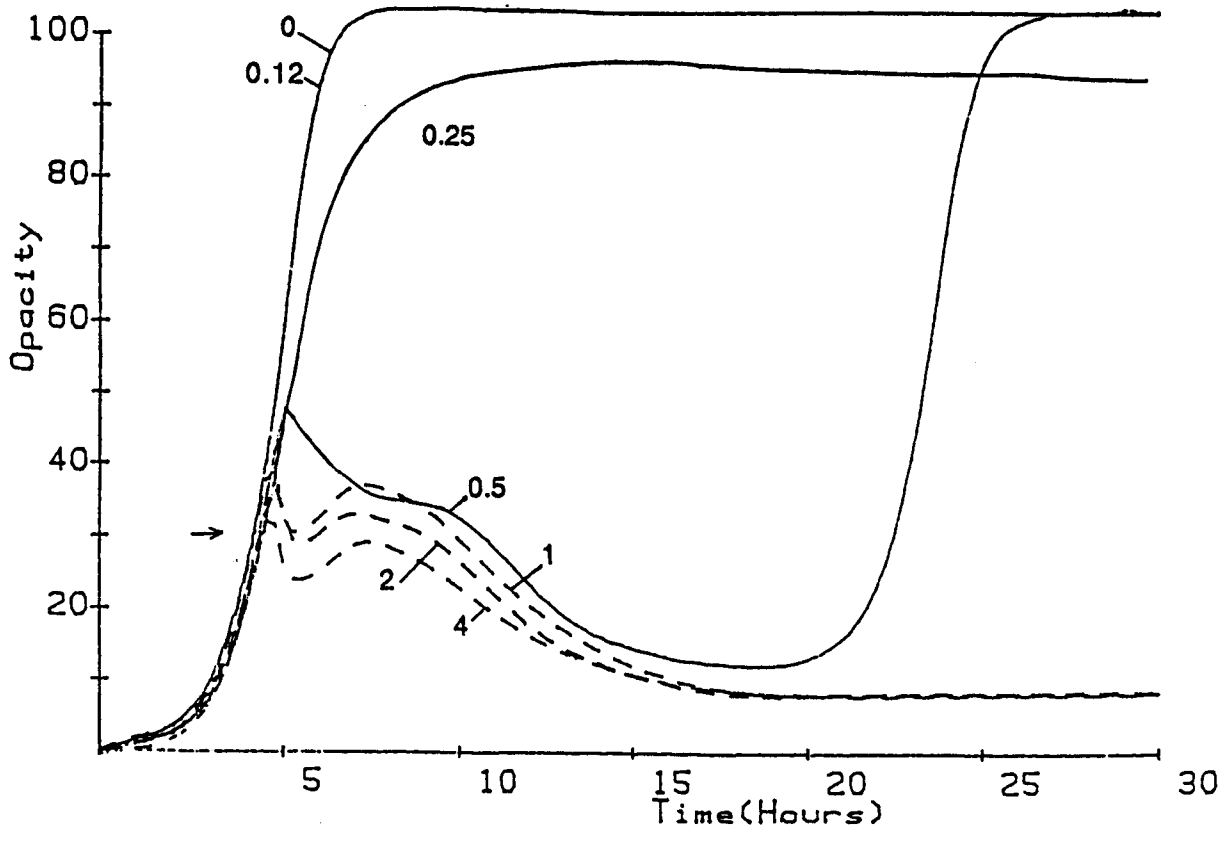


Fig. 5.12 Continuous opacity records of *B.fragilis* R212 exposed at time zero to imipenem at the concentration shown (mg/l). The inoculum was *ca.* 10^5 cfu/ml in each case.

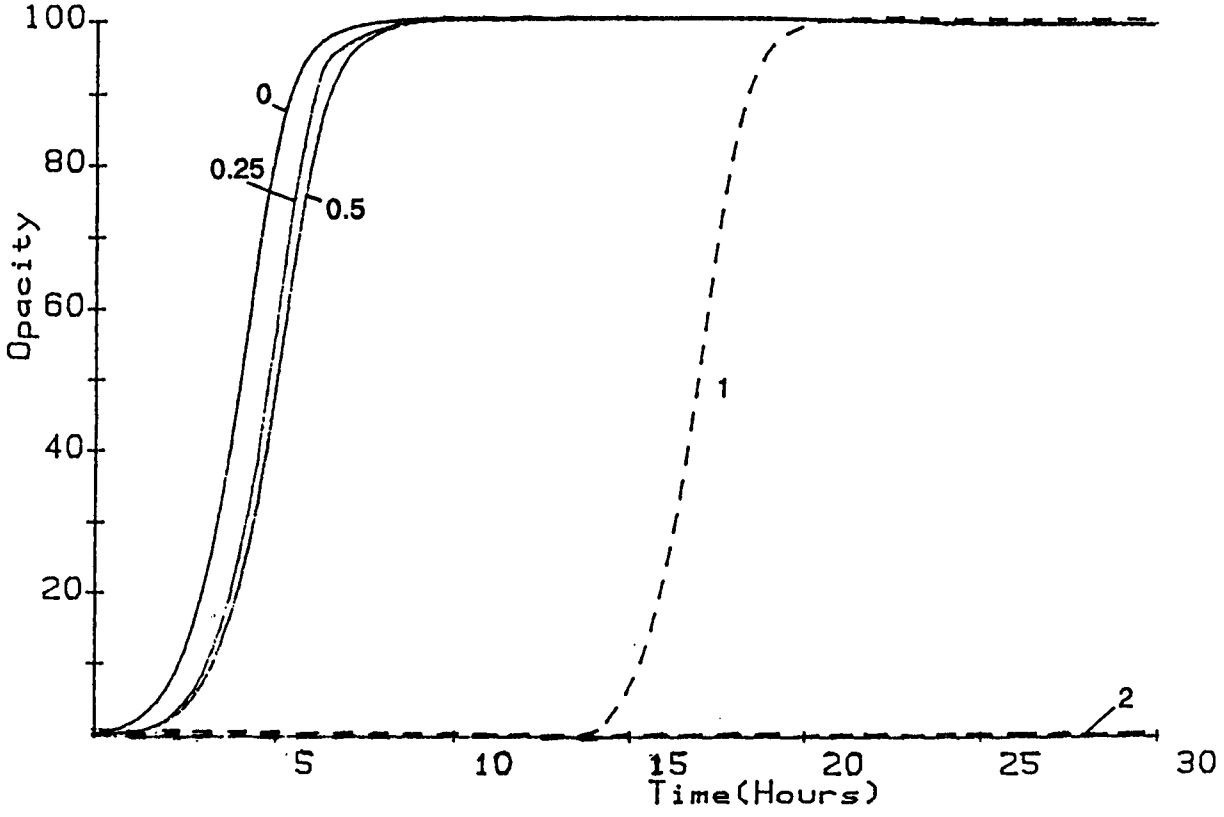


Fig. 5.13 Continuous opacity records of *B.fragilis* R212 with imipenem added at 30% opacity (indicated by arrow) to achieve the concentration (mg/l) shown

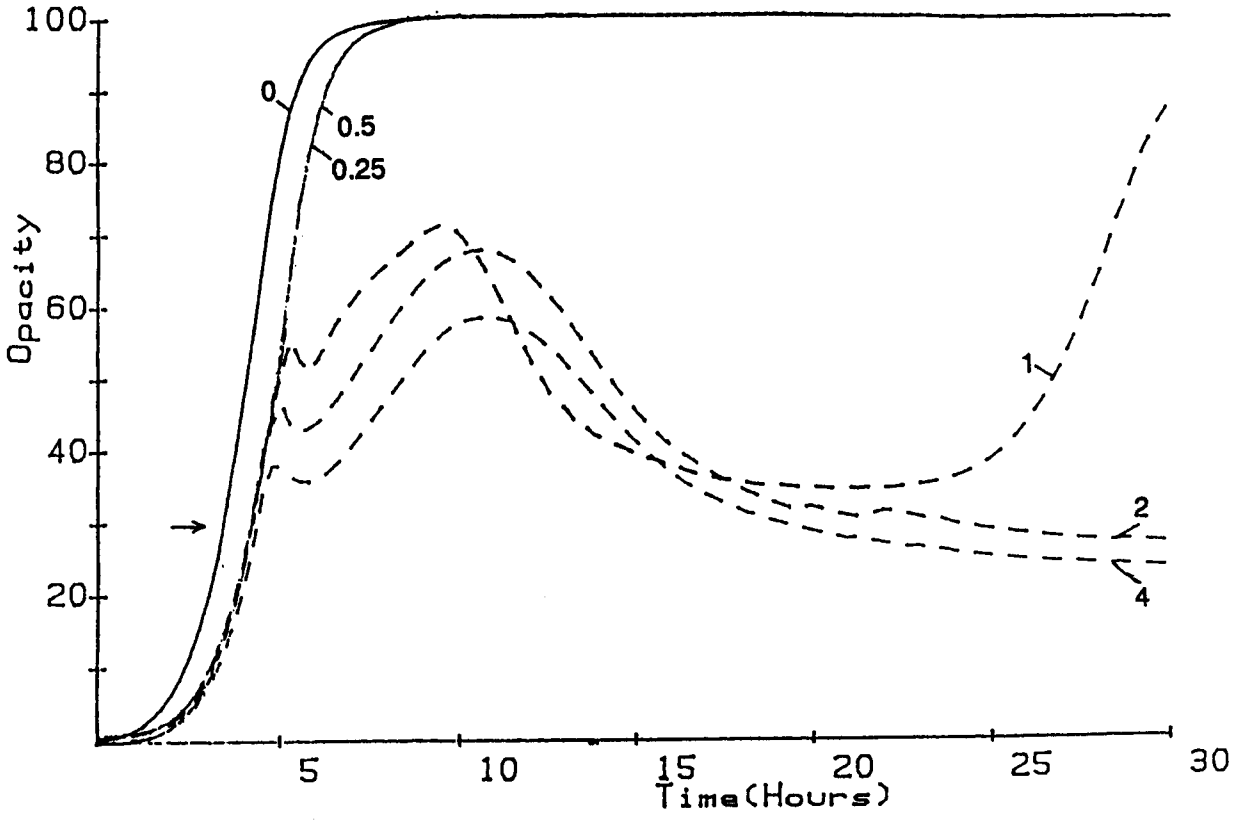


Fig. 5.14 Continuous opacity records of *B.fragilis* 2013E exposed at time zero to imipenem at the concentration shown (mg/l). The inoculum was *ca.* 10^5 cfu/ml in each case.

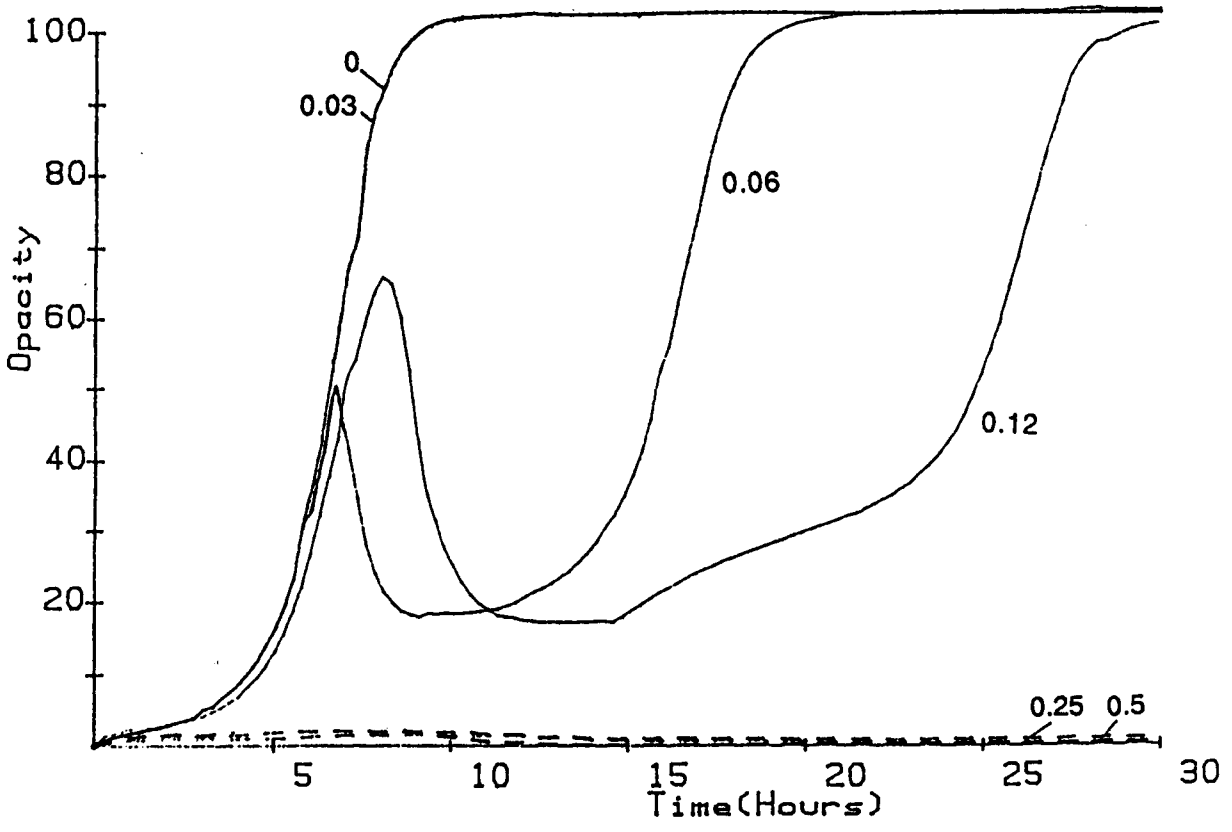


Fig. 5.15 Continuous opacity records of *B.fragilis* 2013E with imipenem added at 30% opacity (indicated by arrow) to achieve the concentration (mg/l) shown

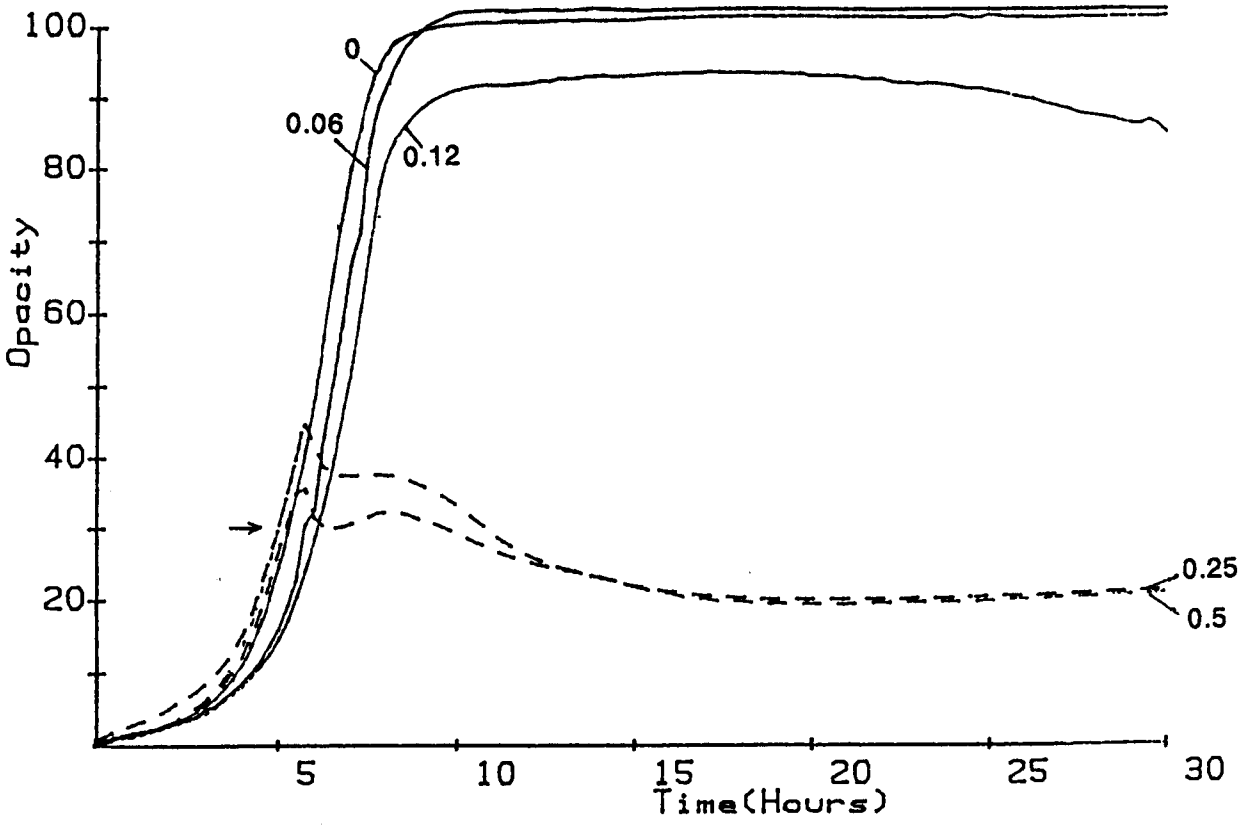


Fig. 5.16 Continuous opacity records of *B.fragilis* 0423 exposed at time zero to imipenem at the concentration shown (mg/l). The inoculum was *ca.* 10^5 cfu/ml in each case.

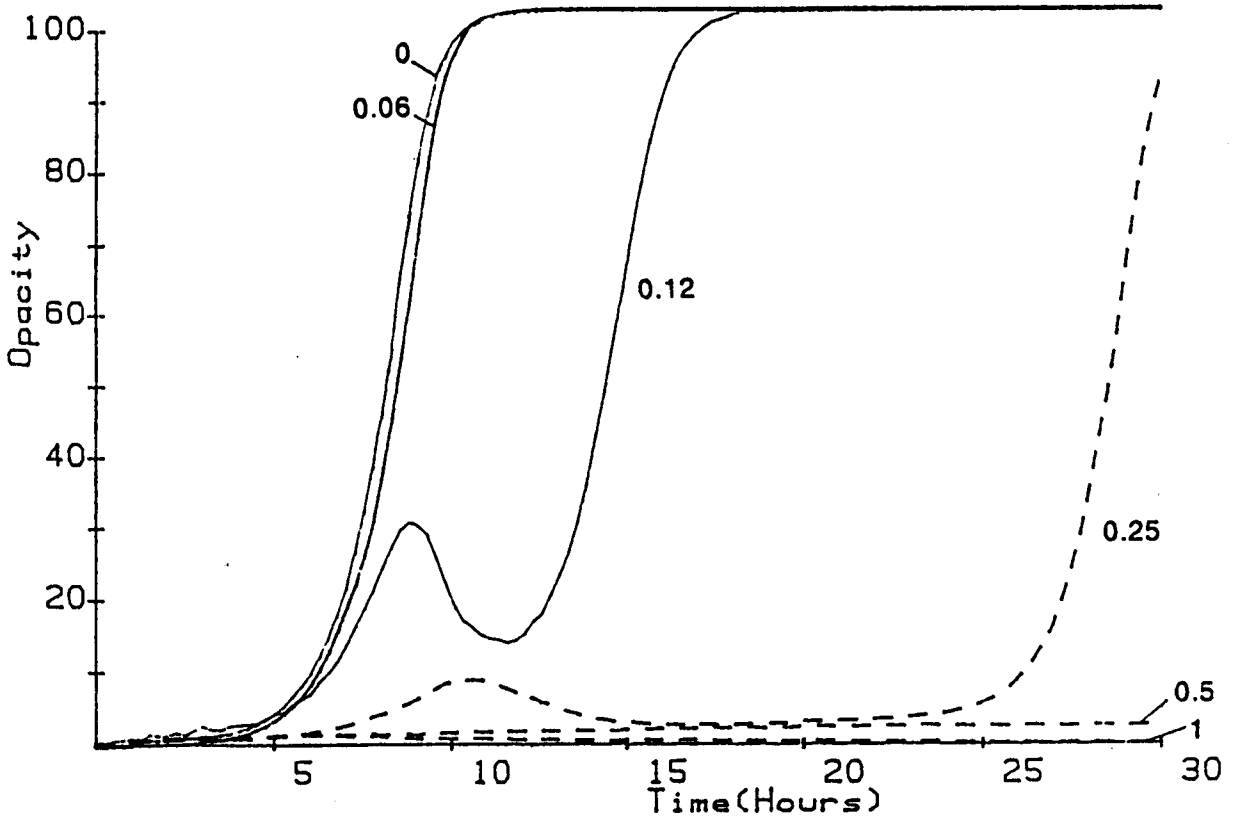


Fig. 5.17 Continuous opacity records of *B.fragilis* 0423 with imipenem added at 30% opacity (indicated by arrow) to achieve the concentration (mg/l) shown

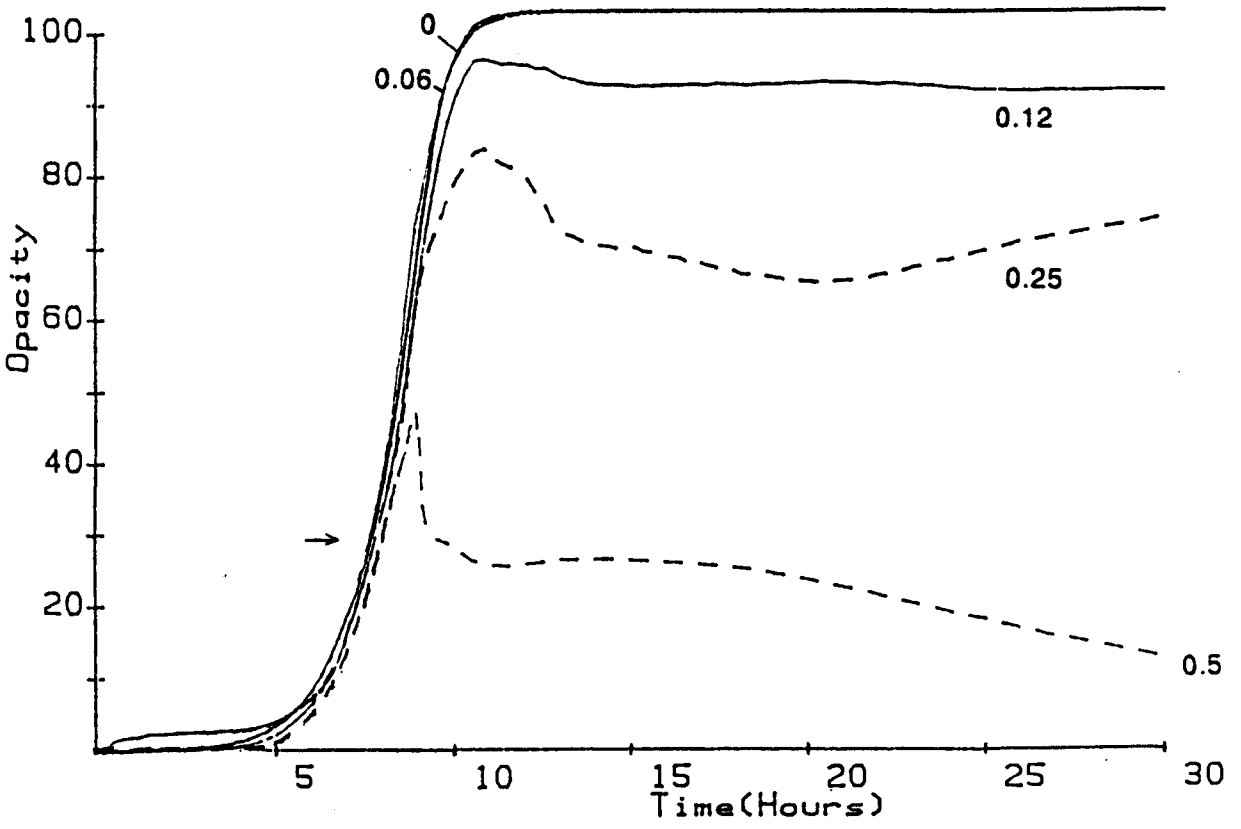


Fig. 5.18 Continuous opacity records of *B.fragilis* NCTC 9344 exposed at time zero to imipenem at the concentration shown (mg/l). The inoculum was *ca.* 10^5 cfu/ml in each case.

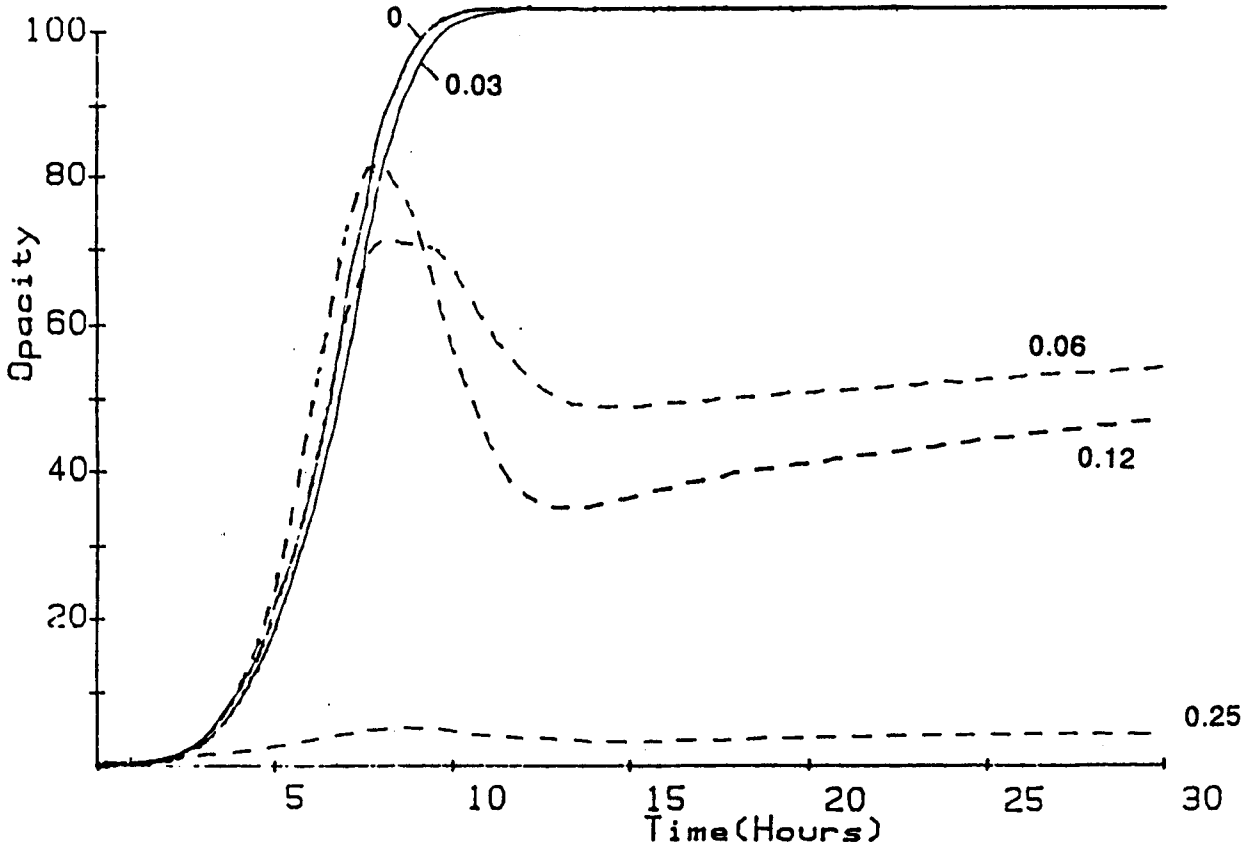


Fig. 5.19 Continuous opacity records of *B.fragilis* NCTC 9344 with imipenem added at 30% opacity (indicated by arrow) to achieve the concentration (mg/l) shown

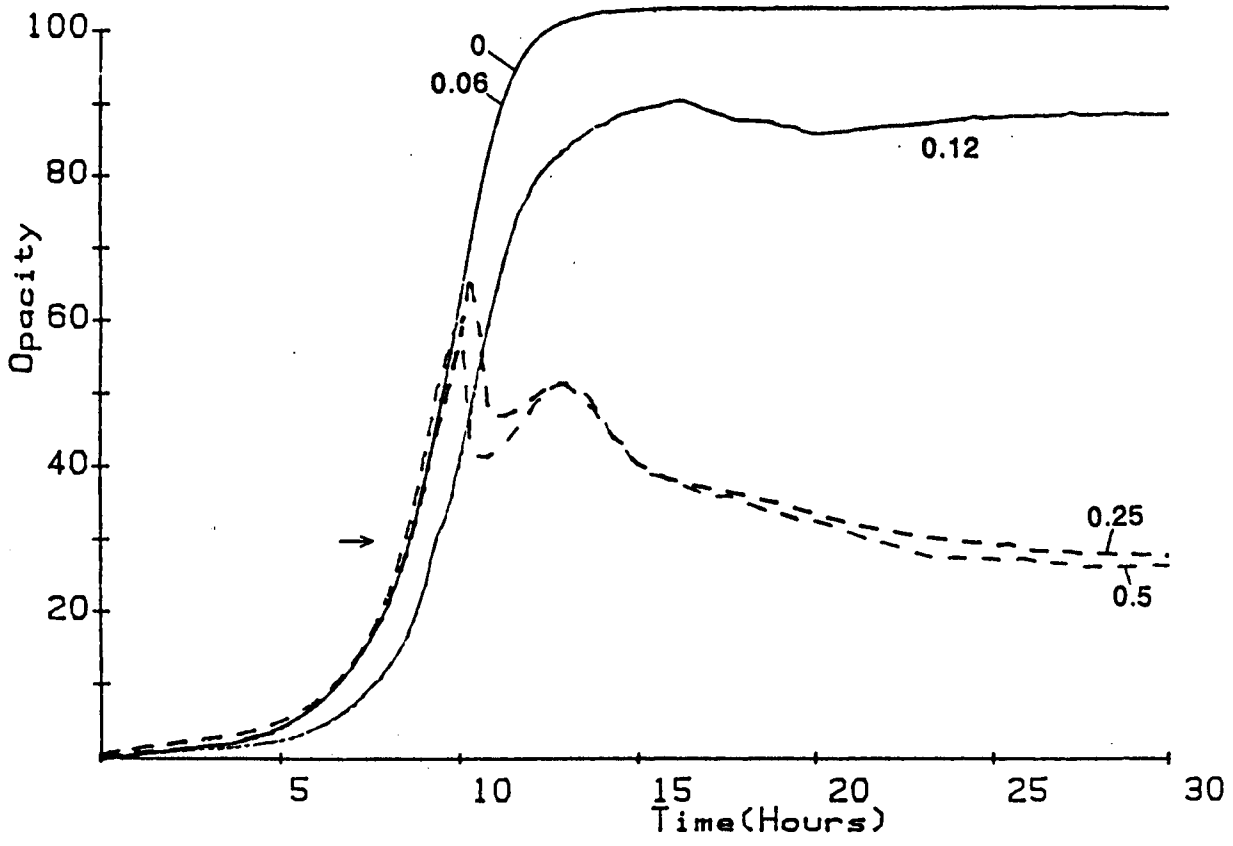


Table 5.3 Summary of turbidimetric data showing the MACs and MICs of imipenem for the high and low inocula of each isolate.

Bacterial strain	inoculum	MAC (mg/l)	MIC (mg/l)
B.fragilis R186	low	≤0.25	2
	high	2	4
B.fragilis R240	low	0.12	2
	high	1	4
B.fragilis R251	low	≤0.25	2
	high	2	4
B.fragilis 119	low	≤1	16
	high	16	64
B.fragilis R208	low	0.12	1
	high	0.25	1
B.fragilis R212	low	1	2
	high	1	2
B.fragilis 2013E	low	0.06	0.25
	high	0.12	0.25
B.fragilis 0423	low	0.12	0.5
	high	0.12	0.5
B.fragilis NCTC 9344	low	0.06	0.25
	high	0.12	0.25

A striking effect noted in experiments in which low inocula of *B.fragilis* R186, R240, R251, 119, 2013E and NCTC 9344 were exposed to imipenem was a marked increase in turbidity that occurred up to 10 hours after the addition of the antibiotic before the onset of cell lysis and subsequent regrowth (Figs. 5.2, 5.4, 5.6, 5.8, 5.14 and 5.18). This initial increase in turbidity before cell lysis was less marked with *B.fragilis* 0423 and R208, and absent with *B.fragilis* R212 (Figs. 5.10, 5.12 and 5.16). These findings could not be associated with any morphological changes and remain unexplained.

5:5 Effect of clavulanic acid on broth dilution MICs of imipenem

The effect of the *B.fragilis* β-lactamases on the activity of imipenem was also investigated by the use of the β-lactamase inhibitor, clavulanic acid. Inhibitor profiles (section 4.4) indicate that the response of β-lactamases of *B.fragilis* test strains to clavulanic acid were diverse: *B.fragilis* R186, R240, R251 and 119 were resistant, *B.fragilis* R208 and R212 showed moderate susceptibility, and *B.fragilis* 0423 and 2013E were fully susceptible. The broth dilution MICs of imipenem, with and without clavulanic acid, are displayed in Table 5.4.

The impact of the β-lactamase inhibitor on the MICs of imipenem for *B.fragilis* R186, R240, R251, 119, R212 and 0423 was minimal, the MICs in the presence of inhibitor being equal to or within one two-fold dilution of the MIC without inhibitor. The negligible effect of clavulanic acid on the sensitivity of imipenem to *B.fragilis* R186, R240, R251 and 119 was to be expected in view of the resistance of the enzymes to this inhibitor. The results with the clavulanic acid

Table 5.4 Broth dilution MICs of imipenem in the presence and absence of clavulanic acid

Bacterial strain	MICs (mg/l) imipenem	imipenem + clavulanic acid*
B.fragilis R186	4	4
B.fragilis R240	4	2
B fragilis R251	4	4
B.fragilis 119	16	8
B.fragilis R208	2	0.5
B.fragilis R212	4	2
B.fragilis 2013E	2	0.25
B.fragilis 0423	2	1
B.fragilis NCTC 9344+	0.12	0.06

* clavulanic acid at a fixed concentration of 4 mg/l
+ control strain

susceptible enzymes of *B.fragilis* R212 and 0423, however, indicated that β-lactamase activity was not responsible for the resistance of these strains.

The MICs of imipenem in the presence of clavulanic acid for *B.fragilis* R208 and 2013E were between four and eight-fold less than those of imipenem alone. Enzymes from these strains had previously shown susceptibility to clavulanic acid, and an increase in imipenem activity in the presence of this inhibitor suggests some involvement of β-lactamases in the increased resistance to imipenem.

For further investigations into the activities and characterisation of carbapenemases, enzymes from three additional *B.fragilis* strains, R249, 57 and 97, known or reported to produce imipenem hydrolysing β-lactamases, were included. The MIC of imipenem for *B.fragilis* R249 from this study was 1 mg/l (section 3:5). *B.fragilis* 57 and 97 were clinical isolates described by Eley and Greenwood (1986a) and the MIC of imipenem for these strains was reported as 8 mg/l and 0.25 mg/l respectively.

5:6 Comparative activity and stability of imipenem and meropenem

The susceptibility and β-lactamase activity of *B.fragilis* which showed increased resistance to imipenem were assessed using meropenem in order to ascertain whether these characteristics determined with imipenem were common to other carbapenems.

Meropenem and imipenem were shown generally to have similar activity against these *B.fragilis* strains, MICs of both compounds being within one dilution for all except three strains,

B.fragilis R240, R249 and 97, all carbapenemase producers, for which the MIC of meropenem was four-fold higher than that of imipenem (Table 5.5).

The degradation of meropenem and imipenem, as measured by HPLC, by identical samples of concentrated crude cell extracts of these *B.fragilis* strains, is given in Figs. 5.20 and 5.21. Hydrolysis of the two carbapenems was similar. Extracts of *B.fragilis* R186, R240, R249, R251, 57, 97 and 119 rapidly hydrolysed both meropenem and imipenem. Hydrolysis of both carbapenems was generally complete within four hours although a small residue of imipenem was still detectable after 20 hours of incubation in the case of *B.fragilis* R186.

5:7 Specific imipenemase activity

The specific imipenemase activity (SIA) was defined as the number of μ moles imipenem hydrolysed/min/mg of protein in the cell extract. Hydrolysis of imipenem was determined spectrophotometrically by a decrease in absorbance at 299nm and 37°C. Not suprisingly, enzymes from *B.fragilis* R208, R212, 2013E, 0423 and NCTC 9344, previously shown to be incapable of rapid hydrolysis of imipenem, showed no or minimal change in absorbance (SIA = 0). With *B.fragilis* strains R186, R240, R249, R251, 57, 97 and 119, the reduction in absorbance was most rapid over the initial five minute period of exposure of imipenem to the β-lactamases. The specific imipenemase activities of these strains are shown in Table 5.6. The greatest specific activities were obtained with *B.fragilis* strains 57 and 119 (0.026 and 0.027 μmoles/min/mg of protein respectively). These values are approximately two to ten times greater than the specific activities of the remaining strains. Of the strains tested, *B.fragilis* 57 and 119 were the least susceptible to imipenem

Table 5.5 Comparative susceptibilities to imipenem and meropenem of eleven *B.fragilis* test strains.

Bacterial	strain	MIC mg/l meropenem	imipenem
B.fragilis	R186	4	2
B.fragilis	R240	8	2
B.fragilis	R249	2	0.5
B.fragilis	R251	4	2
B.fragilis	57	16	8
B.fragilis	97	2	0.5
B.fragilis	119	32	16
B.fragilis	R208	2	2
B.fragilis	R212	2	2
B.fragilis	2013E	2	2
B.fragilis	0423	0.5	1
B.fragilis	NCTC 9344*	0.12	0.12

* control strain

Fig. 5.20 Hydrolysis of imipenem by enzyme extracts from *B.fragilis* strains

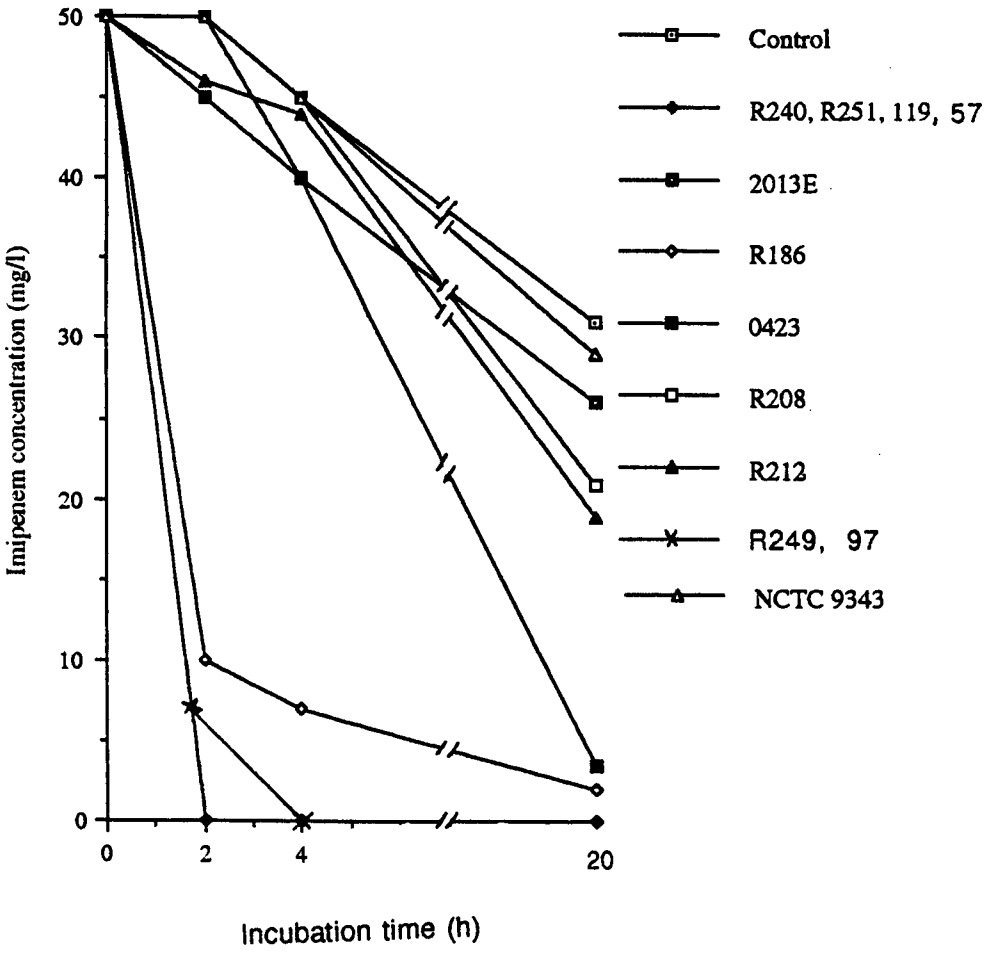


Fig. 5.21 Hydrolysis of meropenem by enzymes from *B.fragilis* strains

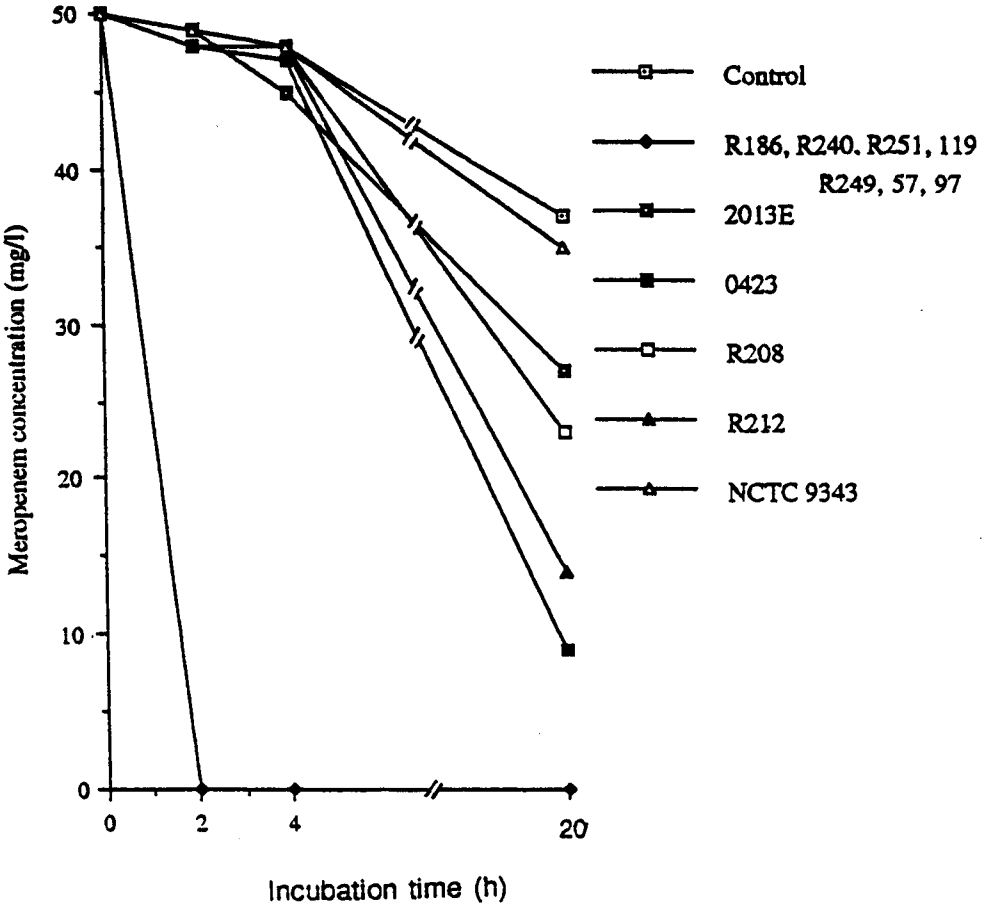


Table 5.6 Specific imipenemase activity of extracts of seven carbapenemase producing *B.fragilis* strains that showed increased resistance to imipenem

Bacterial strain	specific imipenemase activity*
B.fragilis R186	0.003
B.fragilis R240	0.012
B.fragilis R249	0.004
B.fragilis R251	0.008
B.fragilis 57	0.026
B.fragilis 97	0.008
B.fragilis 119	0.027

* μ moles imipenem hydrolysed/min/mg of protein

(MIC 8 mg/l and 16 mg/l respectively).

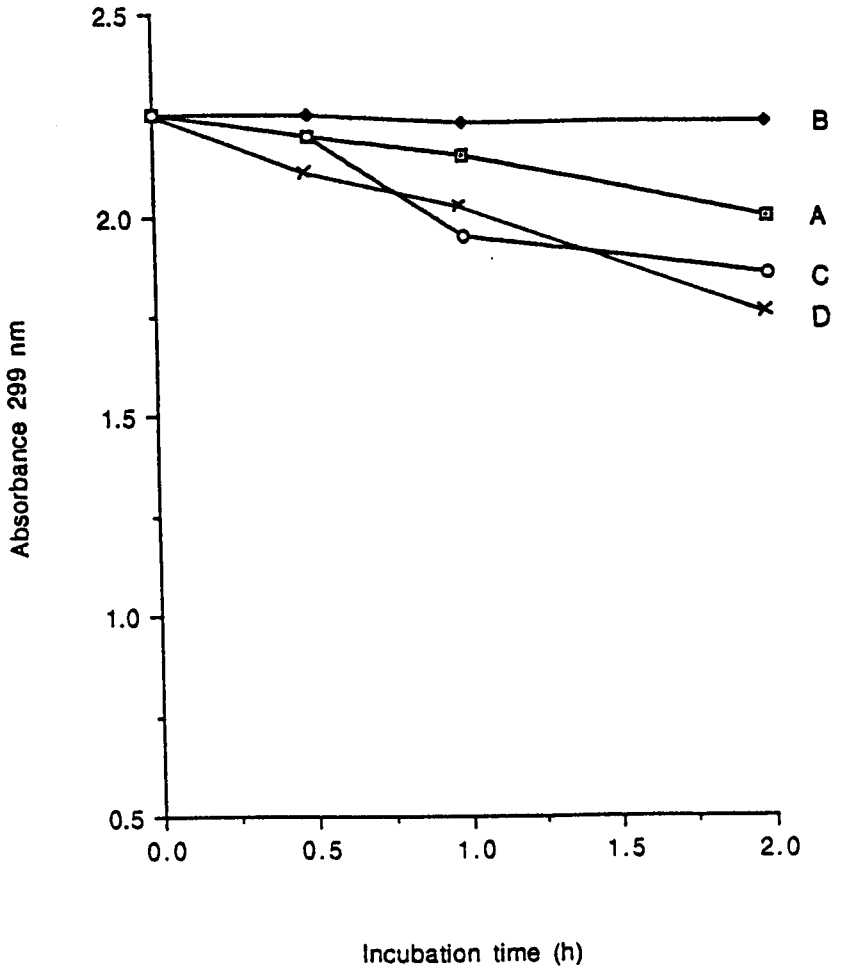
5:8 Detection of metallo-β-lactamases

To examine whether the organisms produced zinc dependent β-lactamases (metallo-β-lactamases), inhibition of hydrolysis of imipenem in the presence of the ion chelator EDTA and the restoration of β-lactamase activity with the addition of zinc ions was investigated. Experiments were also carried out with nitrocefin as substrate to determine the presence of any non-metallo-cephalosporinases in addition to metallo-β-lactamases.

(1) With imipenem as substrate

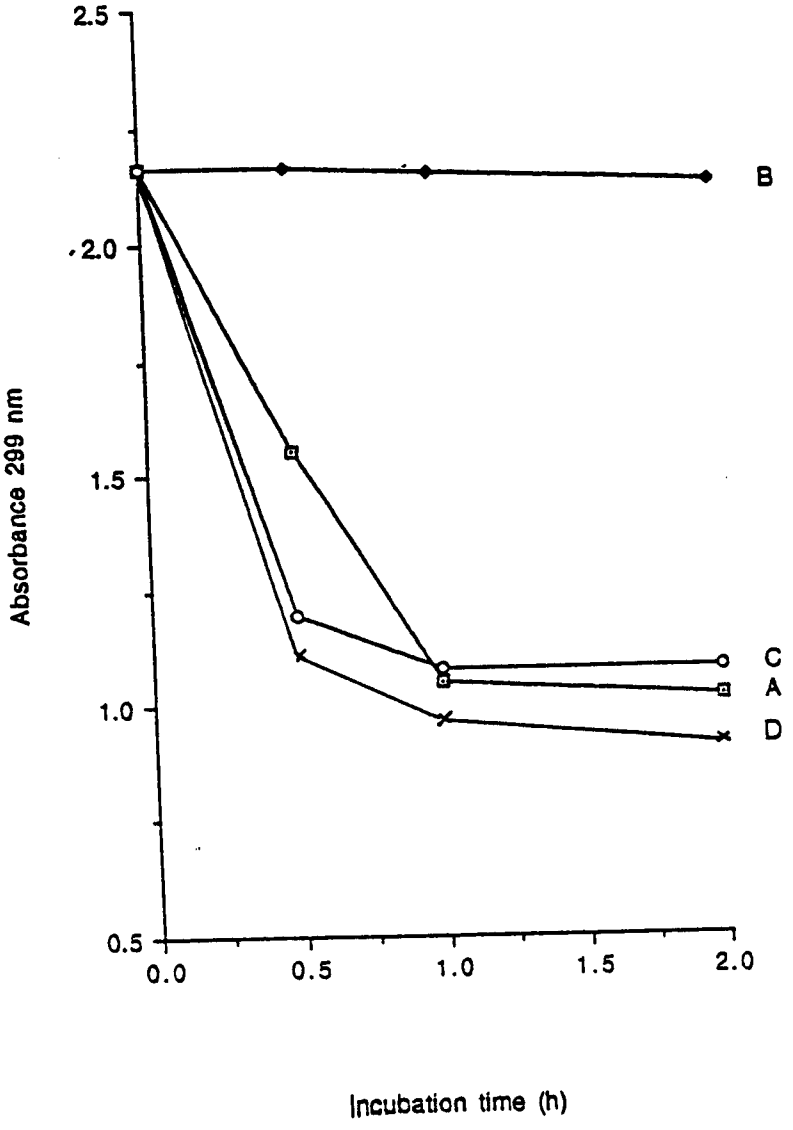
The changes in absorbance at 299 nm and 37°C, representing hydrolysis of imipenem by concentrated crude extracts of bacteroides strains with and without EDTA (1 mM) or EDTA and zinc sulphate (1 mM) or zinc sulphate, are shown in Figs. 5.22-5.28. β-Lactamases from *B.fragilis* R186, R240, R249, R251, 57, 97 and 119 hydrolysed imipenem, although the rate of degradation by *B.fragilis* R186 and R249 was slow. EDTA inhibited imipenem hydrolysis by all these strains, and hydrolysis was restored when zinc sulphate was added. β-Lactamases from these seven strains, therefore, appeared to be zinc dependent carbapenemases or metallo-β-lactamases. There was a tendency for the addition of zinc sulphate to potentiate hydrolysis of imipenem in the presence and absence of EDTA, indicating insufficient zinc ions in the enzyme/imipenem test system to allow full expression of β-lactamase activity. This affect was particularly marked in the case of *B.fragilis* R251. Enzymes from *B.fragilis* R208, R212, 2013E, 0423 and the NCTC 9344 control produced no change in absorbance with or without EDTA indicating their inability to hydrolyse imipenem and the absence of metallo-β-

Fig. 5.22 Spectrophotometric evaluation of the hydrolysis of imipenem by crude enzyme extracts of *B.fragilis* R186 in the presence of ethylenediaminetetraacetic acid (EDTA) and zinc sulphate



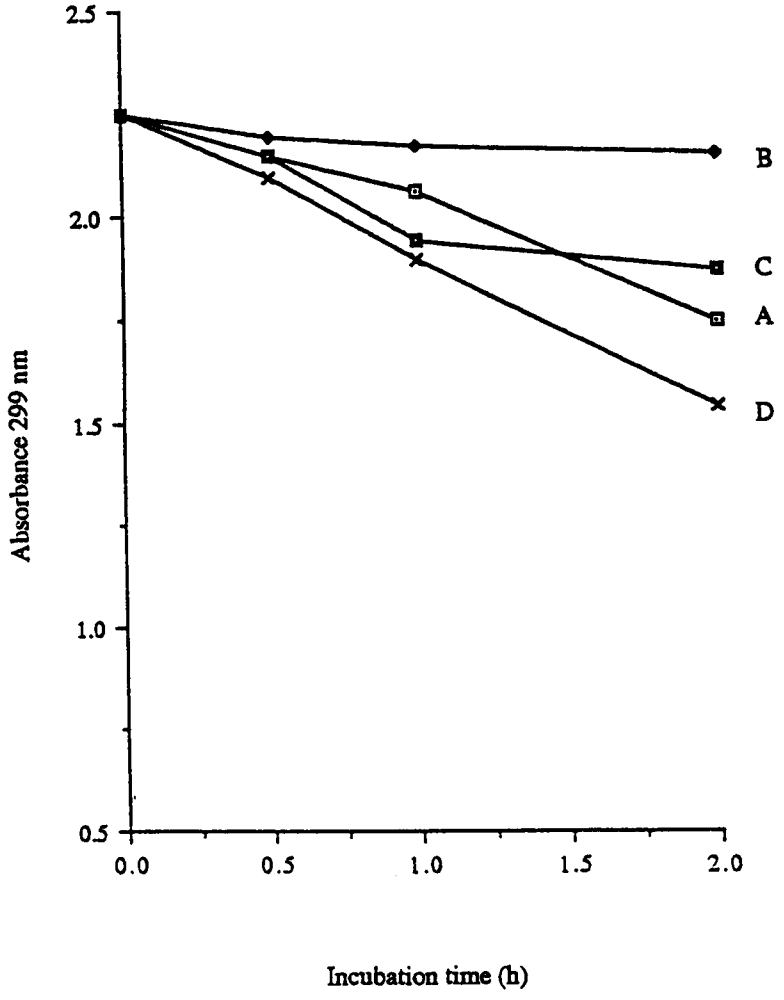
A. phosphate buffer
B. EDTA (1 mM)
C. EDTA (1mM) and zinc sulphate (1mM)
D. zinc sulphate (1mM)

Fig. 5.23 Spectrophotometric evaluation of the hydrolysis of imipenem by crude enzyme extracts of *B.fragilis* R240 in the presence of ethylenediaminetetraacetic acid (EDTA) and zinc sulphate



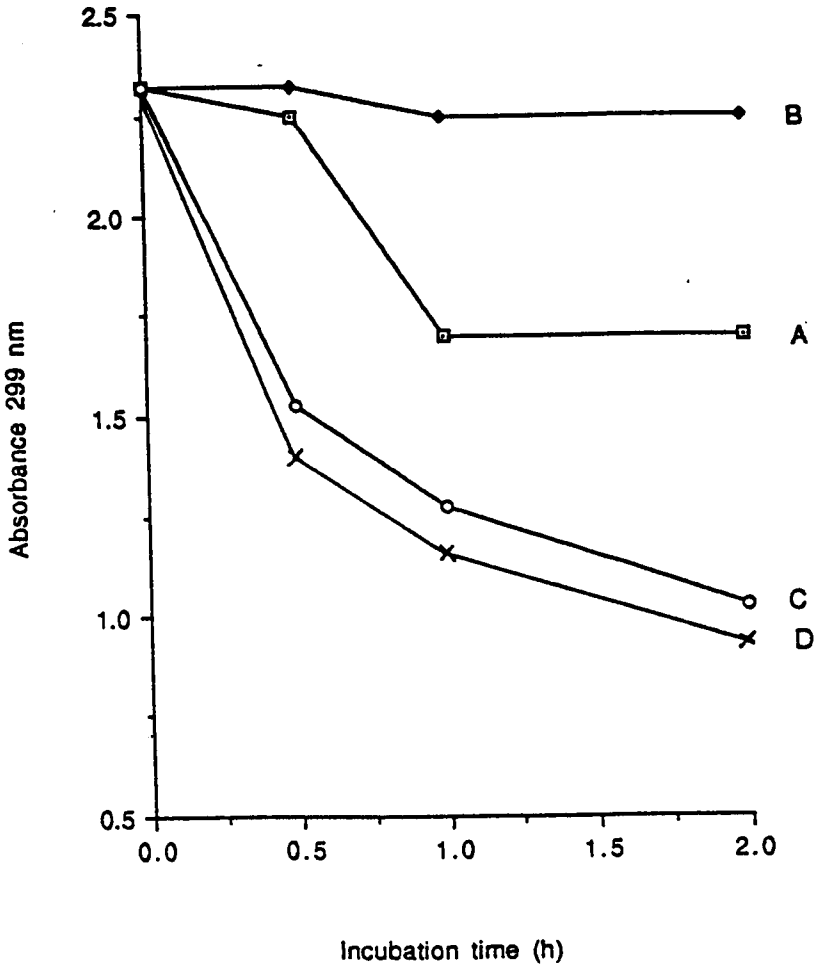
A. phosphate buffer
B. EDTA (1 mM)
C. EDTA (1mM) and zinc sulphate (1mM)
D. zinc sulphate (1mM)

Fig. 5.24 Spectrophotometric evaluation of the hydrolysis of imipenem by crude enzyme extracts of *B.fragilis* R249 in the presence of ethylenediaminetetraacetic acid (EDTA) and zinc sulphate



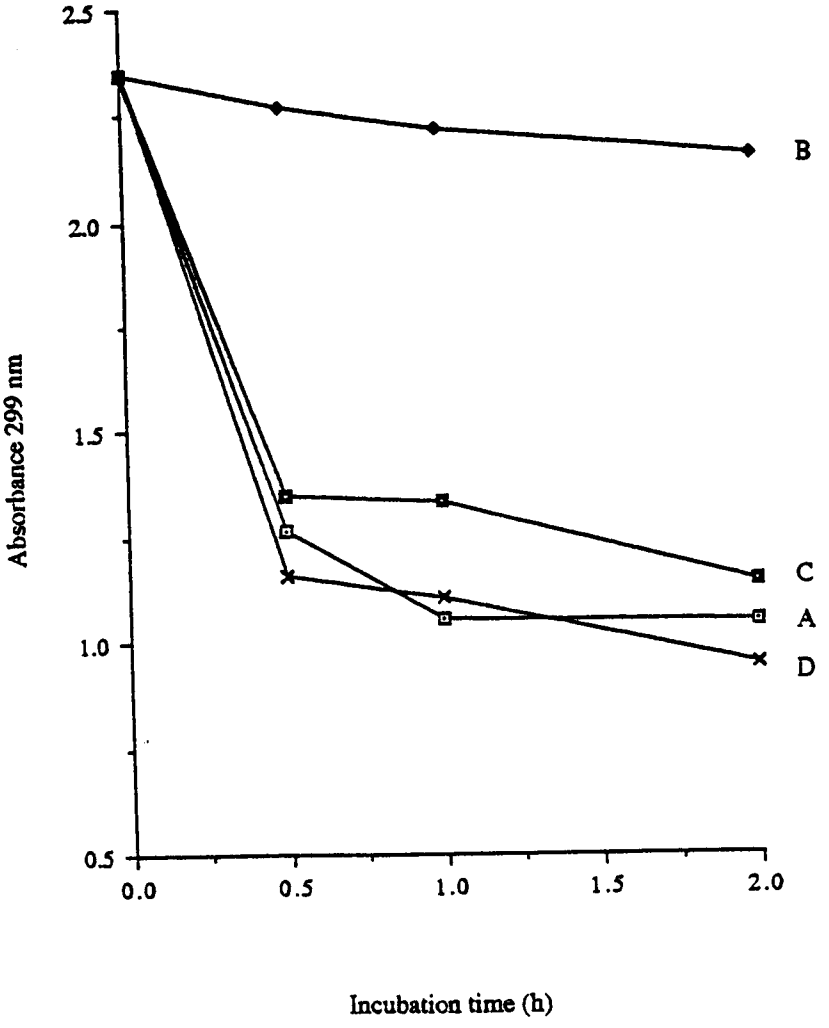
A. phosphate buffer
B. EDTA (1 mM)
C. EDTA (1mM) and zinc sulphate (1mM)
D. zinc sulphate (1mM)

Fig. 5.25 Spectrophotometric evaluation of the hydrolysis of imipenem by crude enzyme extracts of *B.fragilis* R251 in the presence of ethylenediaminetetraacetic acid (EDTA) and zinc sulphate



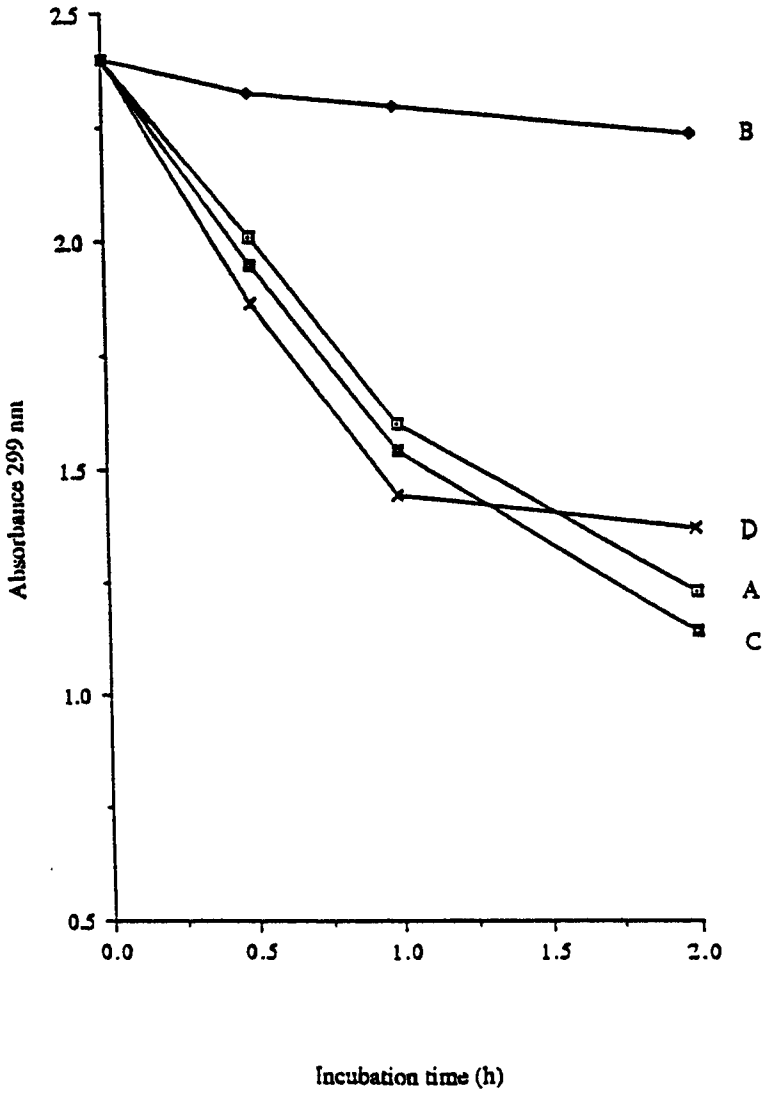
A. phosphate buffer
B. EDTA (1 mM)
C. EDTA (1mM) and zinc sulphate (1mM)
D. zinc sulphate (1mM)

Fig. 5.26 Spectrophotometric evaluation of the hydrolysis of imipenem by crude enzyme extracts of *B.fragilis* 57 in the presence of ethylenediaminetetraacetic acid (EDTA) and zinc sulphate



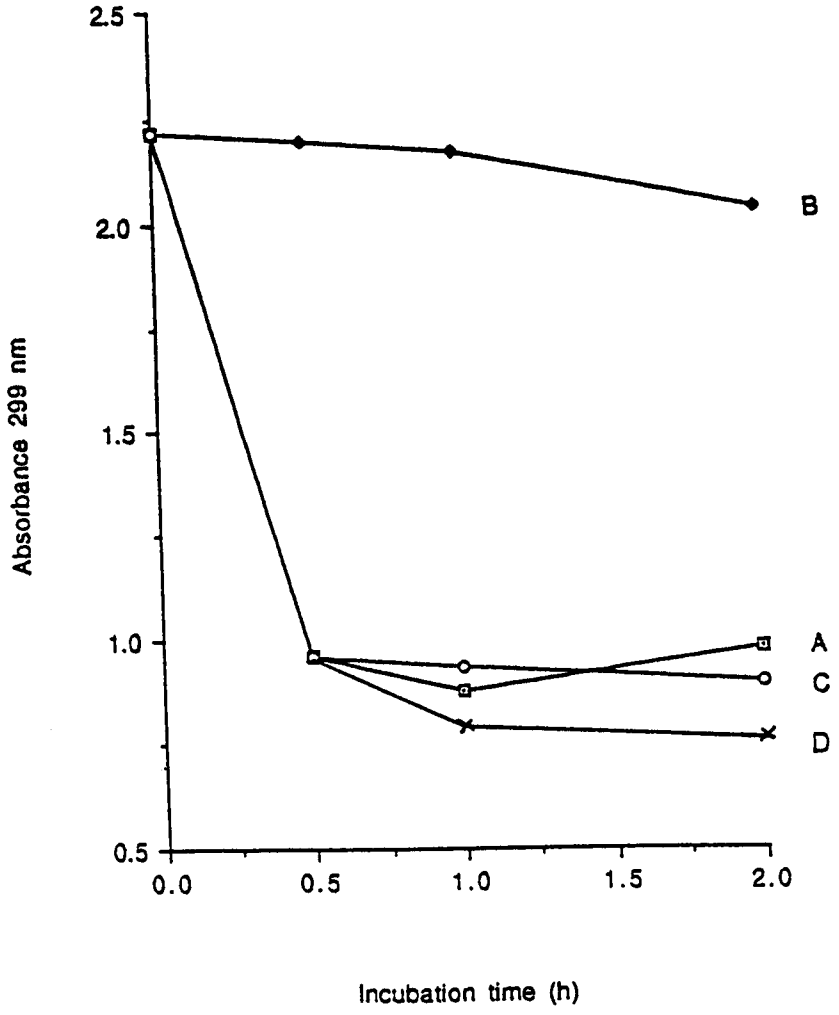
A. phosphate buffer
B. EDTA (1 mM)
C. EDTA (1mM) and zinc sulphate (1mM)
D. zinc sulphate (1mM)

Fig. 5.27 Spectrophotometric evaluation of the hydrolysis of imipenem by crude enzyme extracts of *B.fragilis* 97 in the presence of ethylenediaminetetraacetic acid (EDTA) and zinc sulphate



A. phosphate buffer
B. EDTA (1 mM)
C. EDTA (1mM) and zinc sulphate (1mM)
D. zinc sulphate (1mM)

Fig. 5.28 Spectrophotometric evaluation of the hydrolysis of imipenem by crude enzyme extracts of *B.fragilis* 119 in the presence of ethylenediaminetetraacetic acid (EDTA) and zinc sulphate



A. phosphate buffer
B. EDTA (1 mM)
C. EDTA (1mM) and zinc sulphate (1mM)
D. zinc sulphate (1mM)

lactamases.

(2) With nitrocefin as substrate

The rates of change in absorbance at 482 nm and 37°C in experiments in which nitrocefin was incubated with concentrated crude cell extracts in the presence of phosphate buffer, EDTA (1 mM), zinc sulphate (1 mM) or a mixture of EDTA and zinc sulphate, are shown in Table 5.7. The hydrolysis of nitrocefin by extracts of *B.fragilis* R186, R240, R249, R251, 57, 97 and 119 was completely inhibited by EDTA. With the addition of zinc sulphate, however, hydrolysis of nitrocefin was restored. The results indicate that these strains lack cephalosporin hydrolysing non-metallo-β-lactamases although they produce zinc dependent metallo-β-lactamases. In contrast, non-metallo-cephalosporinases were produced by *B.fragilis* strains R208, R212, 0423 and 2013E, as shown by similar rates of change in absorbance of nitrocefin in buffer, EDTA, and the mixture of EDTA and zinc sulphate. In this system, zinc sulphate alone had no effect on the hydrolysis of nitrocefin by the crude cell extracts tested.

5:9 Effect of zinc acetate on the imipenem susceptibility of carbapenemase producing *B.fragilis*

In order to determine whether the zinc concentration of the BHIS sensitivity testing medium was a factor in the susceptibility to imipenem of isolates of *B.fragilis* which produce zinc dependent carbapenemases, MICs were performed in BHIS with and without supplemented zinc ions. The addition of zinc acetate to the BHIS broth, which increased the zinc ion content from 75 to 150 μmols/l, had minimal effect on the MICs of imipenem of these *B.fragilis* isolates (Table 5.8). This indicates that the imipenem resistance

Table 5.7 Effect of EDTA and zinc sulphate, alone and in combination, on the hydrolysis of nitrocefin by crude extracts of *B.fragilis* strains.

Bacterial strain		Change in absorbance/min (482 nm) of nitrocefin with crude extract and: Buffer	EDTA*	$ZnSO_4$*	EDTA*+$ZnSO_4$*
B.fragilis	R186	0.03	0	0.03	0.03
B.fragilis	R240	0.03	0	0.03	0.03
B.fragilis	R249	0.02	0	0.02	0.02
B.fragilis	R251	0.03	0	0.03	0.01
B.fragilis	57	0.04	0	0.04	0.03
B.fragilis	97	0.02	0	0.03	0.02
B.fragilis	119	0.04	0	0.04	0.04
B.fragilis	R208	0.09	0.10	0.10	0.09
B.fragilis	R212	0.12	0.10	0.10	0.09
B.fragilis	2013E	0.15	0.14	0.12	0.14
B.fragilis	0423	0.12	0.09	0.10	0.09

* at a concentration of 1 mM

Table 5.8 Minimum inhibitory concentration of imipenem of carbapenemase producing *B.fragilis* strains with and without zinc ion supplemented in BHIS.

Bacterial strain	MIC mg/l no supplement[+]	zinc added*
B.fragilis R186	2	2
B.fragilis R240	2	2
B.fragilis R249	0.5	1
B.fragilis R251	2	4
B.fragilis 57	16	16
B.fragilis 97	1	0.5
B.fragilis 119	32	32
B.fragilis NCTC 9344[x]	0.12	0.12

x = non-carbapenemase producing control

+ = final zinc concentration 75μmols/l

* = " " " 150μmols

displayed by these strains when tested in BHIS medium was not artificially low as a result insufficient zinc ions.

5.10 Discussion

Hydrolysis of imipenem was demonstrated by exposure of the drug to both concentrated crude enzyme extracts and whole cells of certain strains of *B.fragilis*. However, the rate of hydrolysis by whole cells was markedly slower than that by concentrated crude enzyme extracts, although the degree of imipenem hydrolysis after 18 - 20 hours incubation was similar. This was despite the possible damaging affects of the sonication process on the intracellular molecular configuration and activity of the β-lactamases present in the crude extracts. The increased rate of hydrolysis by crude cell extracts could be due to the release of the β-lactamases from the periplasmic space and concentration of the enzyme extracts.

Microbiological assay proved more sensitive than HPLC for the measurement of imipenem residues with whole cells and produced levels which tended to be lower than those obtained by HPLC. It is possible that some of the imipenem detected by the HPLC system was not present in a microbiologically active form.

The role of carbapenemases in the resistance to imipenem of *B.fragilis* strains was shown by the inoculum effect observed by turbidimetry and by their specific imipenemase activities. The inoculum effect was particularly marked according to the criterion of a change in the MAC. This effect was minimal with the non-carbapenemase producers. These findings indicate that, in the case of carbapenemase producing strains, increased resistance can be explained entirely by β-lactamase activity. The largest inoculum effect was seen with *B.fragilis* 119; since the MIC of imipenem for this strain

was at least fourfold higher than for the other carbapenemase producing strains, an association between increased levels of carbapenemase and increased resistance to imipenem is implied. Also, the results of experiments to establish the β-lactamase specific imipenemase activities showed that strains which were the most resistant to imipenem exhibited the highest specific activity (*B.fragilis* 57 and 119). Increased carbapenem resistance in *B.fragilis* due to elevated carbapenemase activity has also been reported by Podglajen *et al.* (1990). A correlation between the production by *B.fragilis* of other β-lactamases and their contribution to cephalosporin resistance has previously been established (Olsson *et al.*, 1979; Simpson *et al.*, 1982; Yotsuji *et al.*, 1988; Rogers *et al.*, 1993).

With nitrocefin as substrate, the low levels of hydrolysis observed with the metallo-β-lactamases was completely inhibited by EDTA suggesting the absence of 'normal' 2e type β-lactamases from these strains. Single isoelectric points were found by electrophoresis of extracts of carbapenemase producers including *B.fragilis* R186 (section 4:3), indicating the presence of one β-lactamase type. Yotsuji *et al.* (1983), Eley and Greenwood (1986a,b) and Bandoh *et al.* (1991) described similar findings. Podglajen *et al.* (1992a), however, described metallo-β-lactamase producing *B.fragilis* strains with two species of enzyme as shown by isoelectric focusing. The band remaining after treatment with EDTA, which removed the metallo-β-lactamase activity, had a pI value of ≤4 and is therefore unlikely to represent 'normal' *B.fragilis* β-lactamases for which the pI is *ca.* 5.

The strains examined were, in most cases, equally sensitive to meropenem and imipenem, although three of the seven carbapenemase producers were marginally less susceptible to

meropenem. These carbapenems have been previously shown to exhibit similar activity against unselected clinical isolates of *B.fragilis* and other *Bacteroides* species (Edwards *et al.*, 1989; Neu *et al.*, 1989; Sumita *et al.*, 1989). Enzymes of seven *B.fragilis* test strains hydrolysed the two carbapenems with similar efficiency. Neu *et al.* (1989) and Sumita *et al.* (1989) reported on the stability of meropenem and imipenem to various β-lactamases. They found that both carbapenems were stable to β-lactamases from a variety of Gram negative 'coliform' organisms, *P.aeruginosa*, *Staph. aureus* and typical *B.fragilis* strains, although an enzyme from *Xanthomonas maltophilia* was shown to degrade both meropenem and imipenem, the rate of hydrolysis of imipenem being slightly greater than that of meropenem. This β-lactamase activity was associated with carbapenem resistance.

Four strains from the original study and three of the additional strains were shown to produce zinc dependent metallo-β-lactamases. Only seven other *B.fragilis* strains of this type have been characterised (Hedberg *et al.*, 1992; Payne, 1993). The level of resistance was low: MICs of imipenem were usually at or below the breakpoint of 4-8 mg/l recommended to designate resistance (Finegold, 1989; Phillips *et al.*, 1992) although they were 5 to 10 fold greater than values for fully sensitive *B.fragilis* strains. The highest MIC observed was 16 mg/l, considerably lower than the values described for most carbapenemase producing *B.fragilis* strains examined by other workers. High resistance to imipenem in *B.fragilis*, with MICs >100 mg/l, was reported by Cuchural *et al.* (1986b), Ajiki *et al.* (1991), Bandoh *et al.* (1991, 1992) and Hedberg *et al.* (1992), although the MIC of imipenem for an isolate presumed

to produce a metallo-β-lactamase described by Yotsuji *et al.* (1983) was 12.5 mg/l.

The low MICs of imipenem for zinc dependent metallo-β-lactamase producing *B.fragilis* strains in this study did not appear to be due to lack of zinc ions in the sensitivity testing medium. When the MICs of imipenem for these strains were tested in the BHIS broth medium supplemented with zinc acetate to increase the zinc content from 75 μmols/l to 150 μmols/l, no rise in MICs were detected. Failure to demonstrate an effect of zinc ions on the imipenem susceptibility contrasts with the result of experiments with zinc dependent β-lactamase-producing *X. maltophilia* described by Hawkey *et al.* (1993).

Bacteroides were considered to display increased resistance to imipenem if the MIC was ≥2 mg/l; higher than the MIC_{90} for imipenem. Two strains, *B.fragilis* R249 and 97, have been shown to possess imipenem hydrolysing metallo-β-lactamases and yet their MICs of 0.5 mg/l imipenem were within the sensitive range. Shannon *et al.* (1986) reported imipenem-hydrolysing activity in *Aeromonas hydrophila* which did not confer resistance to the drug. They speculated that imipenem may saturate the preferred PBP target at low concentrations so that the β-lactamase was unable to give much protection. This present study shows that low carbapenemase activity is associated with lack of imipenem resistance in *B.fragilis*.

Similar observations have been made recently by Podglajen *et al.* (1992a), who reported four clinical isolates from French hospitals with MICs of 1 mg/l imipenem. These strains carried the carbapenemase gene and enzyme extracts exhibited specific imipenemase activities of *ca.* 3 μmols/min/mg. protein. They were able to produce high resistance to imipenem (MIC >100 mg/l) in these

isolates by one-step mutation on medium containing imipenem at a concentration of 20 mg/l. Enzymes from these mutants displayed a 41 to 95-fold increase in specific imipenemase activity, associated with greater expression of the carbapenemase gene. The isolates of *B.fragilis* investigated in the present study appear to differ from those used by Podglajen *et al.* (1992a) in that the specific imipenemase activities of their enzymes were >100-fold higher. The comparison of specific activities between laboratories can, however, be problematic as results depend on non standardised technical factors such as the degree of centrifugation of the sonicated extracts and growth conditions of bacteria before harvesting (Edwards and Greenwood, 1990).

The effect of clavulanic acid on the activity of imipenem indicated a possible role of β-lactamases other than metallo-β-lactamases in the resistance of *B.fragilis* R208 and 2013E. The degree and rate of hydrolysis of imipenem by cell extracts and whole cells of these strains (See sections 5.2 and 5.3) was, however, only modest. These strains also showed little inoculum effect in turbidimetric experiments, the MACs of imipenem increasing only by a factor of two when the inoculum density was increased (See section 5:4). Although clavulanic acid was shown to inhibit the enzymes produced by these strains, the MICs of imipenem remained above the normal sensitive range in the presence of the inhibitor, implying the involvement of resistance mechanisms other than β-lactamase. It has previously been stated that *B.fragilis* 2013E produces increased amounts of the typical *B.fragilis* chromosomal β-lactamase and *B.fragilis* R208 in many ways resembles this strain (See section 4:8). It is possible, that, in the case of these two organisms, the production of high levels of 'normal' *B.fragilis* β-lactamases, which can slowly

degrade the antibiotic, contributes to reduced bacterial susceptibility. Livermore (1993) also described trace carbapenemase activity in some serine β-lactamases from *P.aeruginosa* and *Enterobacter cloacae*, which, in association with impermeability, could produce resistance. These 'weak' carbapenemases can hydrolyse one or two molecules of carbapenem per minute, but hydrolyse cephalosporins significantly more rapidly. In contast, metallo-β-lactamases hydrolyse carbapenems and other β-lactams at the rate of several molecules per minute.

CHAPTER 6

PERMEABILITY AS A FACTOR IN THE RESISTANCE OF *B.FRAGILIS* TO IMIPENEM

CHAPTER 6

PERMEABILITY AS A FACTOR IN THE RESISTANCE OF *B.FRAGILIS* TO IMIPENEM

6:1 Introduction

B.fragilis R208, R212, 0423 and 2013E displayed reduced susceptibility to imipenem in the absence of imipenem hydrolysing metallo-β-lactamases. Reduction in antibiotic susceptibility can result from an outer membrane (OM) permeability barrier restricting antibiotic penetration into the bacterial cell, although this is rarely the sole factor in resistance (Wexler, 1991; Rasmussen *et al.*, 1993). Decreased OM permeability in *B.fragilis* has been found in combination with the production of β-lactamase or alteration in penicillin-binding proteins (Cuchural *et al.*, 1986b; Piddock and Wise, 1987)

Permeability barriers may be detected indirectly by crypticity measurements, the ratio of the rate of hydrolysis of a susceptible β-lactam by disrupted cells to that of intact cells. High crypticity values indicate decreased permeability. This assumes that cell bound β-lactamases do not behave in a manner markedly differently to that of free enzyme.

Differences in the OM protein and lipopolysaccharide (LPS) composition among sensitive and resistant bacteria of the same species may reflect changes in permeability due to alteration of porin proteins or hydrophobicity (Godfrey *et al.*, 1984; Piddock and Wise, 1987). In order to establish the OM composition of *B.fragilis* strains of normal susceptibility, three clinical isolates (*B.fragilis* R116, R135

and R192) together with a type culture strain (*B.fragilis* NCTC 9344), were investigated. Outer membrane protein and LPS compositions were compared with those observed in isolates which showed reduced susceptibility to imipenem.

6:2 Crypticity measurements

Crypticity values are inversely related to the degree of permeability and are greater than one when the drug permeation is the rate limiting step for β-lactam hydrolysis. Spectrophotometric tests were carried out and nitrocefin was used as the β-lactam substrate, as all of the test strains to be compared were capable of hydrolysing this compound. β-Lactamase crypticity measurements for eight strains that exhibited reduced susceptibility to imipenem are given in Table 6.1. Raised crypticity values of 9 and 6 were exhibited by *B.fragilis* R212 and 0423 respectively, suggesting the presence of a permeabilty barrier in these two strains. Low crypticity ratios of 3 and 2 were observed with *B.fragilis* 119 and R251 respectively. For four of the strains there was no evidence of any permeability barrier (crypticity ratio = 1).

6:3 Outer membrane protein analysis

The OM protein profiles, obtained by SDS PAGE, are shown in Figs. 6.1. All strains tested possessed a major OM protein of molecular weight 44 kDa and other prominent bands of 62 kDa, 42 kDa, 38 kDa, 29 kDa and 22 kDa. The OM profiles of *B.fragilis* R208, R212, 0423 and 2013E were similar to those of the sensitive control strains (*B.fragilis* R135 and NCTC 9344). However, differences were apparent with metallo-β-lactamase producers *B.fragilis* R251, R186, R240 and 119, these four strains showing an extra band of 40 kDa.

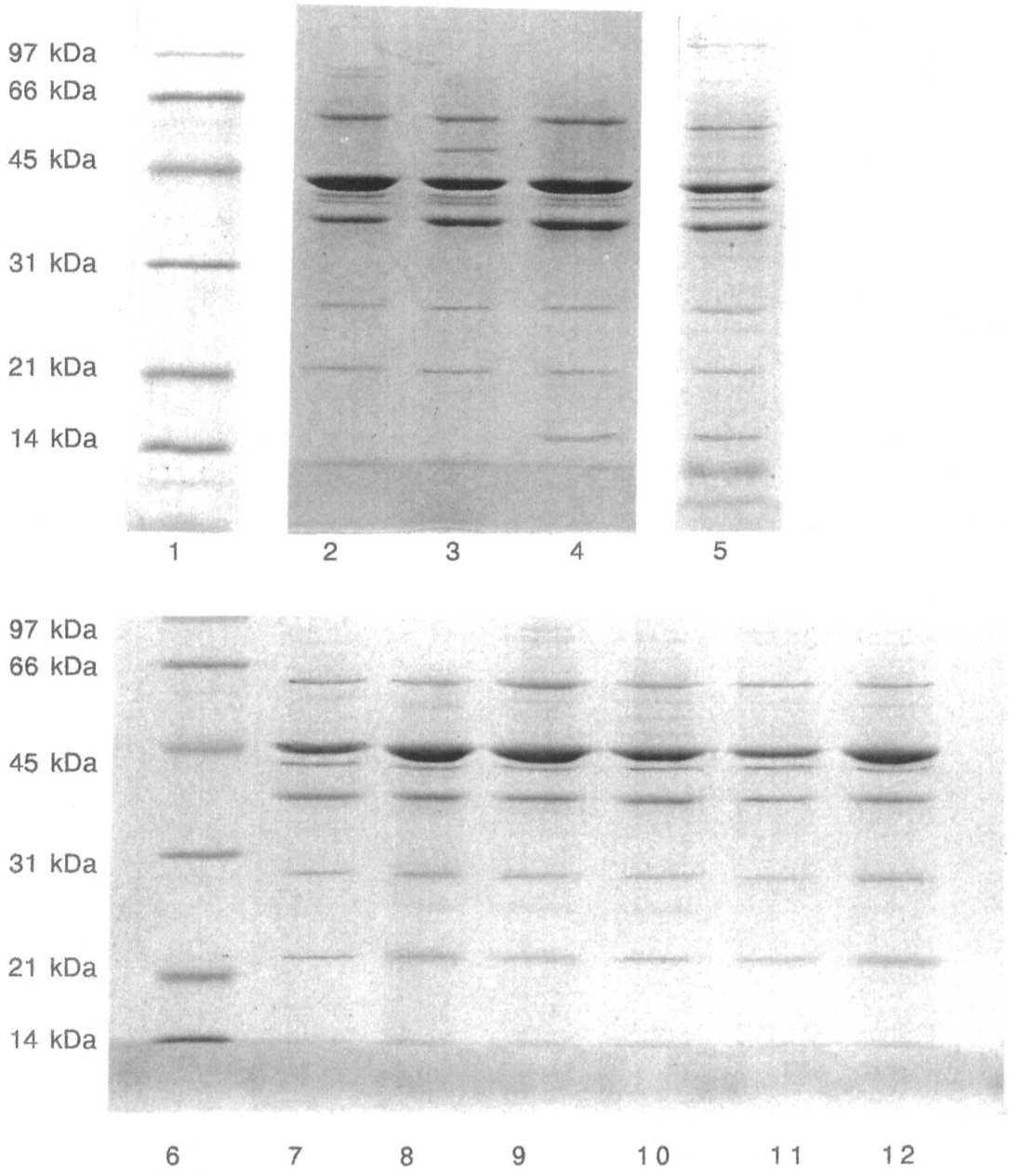
Table 6.1 β-Lactamase crypticity values[#] of *B.fragilis* strains with increased resistance to imipenem

Bacterial strain	MIC (mg/l) imipenem	imipenem hydrolysis	Crypticity values
B.fragilis R186	2	+	1
B.fragilis R240	4	+	1
B.fragilis R251	2	+	2
B.fragilis 119	16	+	3
B.fragilis R208	4	-	1
B.fragilis R212	2	-	9
B.fragilis 2013E	2	-	1
B.fragilis 0423	2	-	6
B.fragilis NCTC 9344*	0.12	-	1

* control strain

[#] the ratio of activity of disrupted cells to that of intact cells

Fig. 6.1 SDS-polyacrylamide gel electrophoresis of outer membrane proteins of *B.fragilis* strains



Lanes: 1 and 6, molecular weight standards; 2, *B.fragilis* R251; 3, *B.fragilis* R186; 4, *B.fragilis* R240; 5, *B.fragilis* 119; 7, *B.fragilis* R212; 8, *B.fragilis* 0423; 9, *B.fragilis* R208; 10, *B.fragilis* 2013E; 11, *B.fragilis* R135; 12, *B.fragilis* NCTC 9344

An additional band of 51 kDa was seen in the profile of *B.fragilis* R186. *B.fragilis* R240 and 119 showed a distinctive 16 kDa protein.

6:4 Lipopolysaccharide analysis

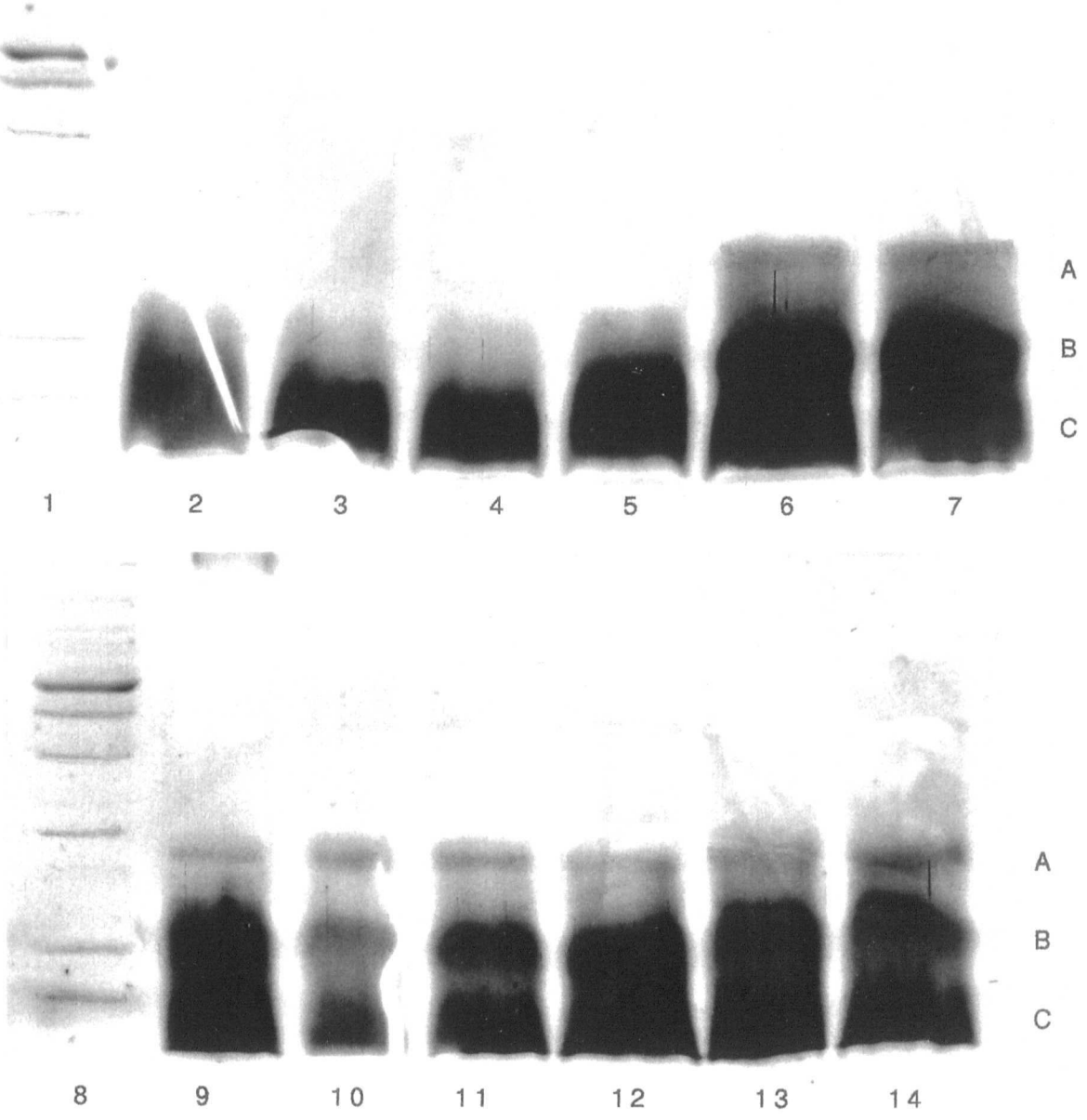
The outer membrane LPS profiles, revealed by silver stained polyacrylamide gels, are shown in Figs. 6.2. The control strains *B.fragilis* R116, R135, R192 and NCTC 9344 displayed an OM LPS composition similar to that of *B.fragilis* R208, R212, 0423 and 2013E, with a major component (band B) and other bands of higher (band A) and lower (band C) molecular weights. However, LPS of band A was not present in *B.fragilis* R251, R186, R240 and 119 and band B was either absent or more diffuse in these strains. All three LPS bands were present at the base of the gels, adjacent to the low molecular weight markers, indicating the presence of 'rough' LPS, with short or absent polysaccharide side chains.

6:5 Discussion

Imipenem appears to be generally highly efficient at penetrating Gram negative cells. In *E.coli* K-12, zwitterionic β-lactam compounds have been shown to penetrate rapidly, and imipenem showed the highest permeability of these compounds due, in part, to its compact molecular structure (Yoshimura and Nikaido, 1985). However, decrease in OM permeability and changes in OM proteins have been implicated in imipenem resistance in *P. aeruginosa* and *Enterobacter aerogenes* (Buscher *et al.*, 1987; Trias *et al.*, 1989; Chow and Shlaes, 1991).

Crypticity has been used by Cuchural *et al.* (1986b) to identify a barrier to imipenem permeation in two imipenem resistant strains of *B.fragilis* which produce β-lactamases capable of imipenem

Fig 6.2 Polyacrylamide gel electrophoresis of lipopolysaccharide preparations of *B.fragilis* strains



Lanes: 1 and 8, molecular weight markers; 2, *B.fragilis* R251; 3, *B.fragilis* R186; 4, *B.fragilis* R240; 5, *B.fragilis* 119; 6, *B.fragilis* NCTC 9344; 7, *B.fragilis* R192; 9, *B.fragilis* R212; 10, *B.fragilis* 0423; 11, *B.fragilis* R208; 12, *B.fragilis* 2013E; 13, *B.fragilis* R135; 14, *B.fragilis* R116
Bands indicated by A, B and C

hydrolysis. The crypticity values for these two strains, with imipenem as substrate, were 14 and 18. In the same study, when nitrocefin was used as substrate, higher crypticity values of 35 and 40 were observed for these strains. This difference may, in part, be due to the greater molecular size of nitrocefin resulting in reduced permeation. Darland and Birnbaum (1977), in a study of cephalosporins, stated that if β-lactamase activity in sonicated extracts increased by a factor of two or less compared to whole cells, the antibiotics are likely to be freely accessible to the enzymes in intact cells. In their study, none of the clinical isolates of *B.fragilis* examined showed a permeability barrier by this criterion. Other studies have correlated crypticity values with β-lactam resistance in *B.fragilis* strains with cephaloridine as substrate. Olsson *et al.* (1979) found crypticity values of greater than one in all five highly resistant *B.fragilis* strains tested; two of these strains showed high crypticity values of 5.5 and 7.3. Timewell *et al.* (1981) reported that almost all *B.fragilis* isolates, with sufficient enzyme for crypticity to be assessed, had a permeability barrier (crypticity >1). The normal value given was 3.5, and in one strain the barrier appeared to account for much of the resistance with a crypticity value of 43.

Although resistance to imipenem and permeation of the antibiotic were under consideration in this study, the crypticity values for nitrocefin, a substrate common to β-lactamases of all the test strains, were assessed to give a crude indication of a general permeability barrier. The highest crypticity values of 9 and 6 were shown with *B.fragilis* R212 and 0423 respectively. These strains exhibited a non-enzymic reduction in susceptibility to imipenem and the results suggest the presence of a permeability barrier which may

be a factor in increased resistance to imipenem. In contrast to the findings of Cuchural *et al.* (1986b), no high crypticity values were detected among the metallo-β-lactamase producers in this study. However, *B.fragilis* 119 gave a crypticity value of 3, possibly indicating a partial barrier to antibiotic passage, and this may, together with the production of β-lactamase of high specific imipenem activity, be a factor in the moderate level of resistance of this strain. Similarly *B.fragilis* R251, also a metallo-β-lactamase producer, showed a low crypticity value of 2, suggesting minor involvement of a pemeability barrier in imipenem resistance. In other test strains, the metallo-β-lactamase producers *B.fragilis* R186 and R240, and the non-metallo-β-lactamase producers *B.fragilis* R208 and 2013E, no evidence was found that associated reduced susceptibility to imipenem with restricted passage of antibiotic into the bacterial cells.

The effect of EDTA on susceptibility is an additional means of examining antibiotic penetration into the bacterial cell. EDTA increases cell permeability and a comparison of sensitivity with and without EDTA reflects antibiotic permeation (Malouin and Lamothe, 1987). This method was considered unsuitable for the examination of *B.fragilis* strains in this study as EDTA also has the ability to remove zinc ions, and this would restrict the activity of the metallo-β-lactamases affecting bacterial susceptibility. Low zinc concentrations in sensitivity testing media has been shown by Hawkey *et al.* (1993) to result in increased susceptibility of metallo-β-lactamase producing *X.maltophilia* strains to imipenem. Zinc is also known to affect the imipenem sensitivity of non-metallo-β-lactamase producing *P.aeruginosa* (Cooper *et al.*, 1993). These workers speculated that zinc could alter the interaction of impenem with penicillin binding proteins. Therefore, the affect of EDTA on bacterial sensitivities may

not be due solely to increased permeability.

Poxton and Brown (1979) found that the OM and 'associated proteins' of several strains of *B.fragilis* were similar to each other yet distinct from those of several other species of *Bacteroides*. The OM protein profiles obtained with the sensitive *B.fragilis* strains tested, including the *B.fragilis* NCTC 9344 control, appeared similar to that of the sensitive *B.fragilis* ATCC 25285 strain described by Diedrich and Martin (1981), which had a major protein band of 43 kDa.

The similarity in OM protein profiles between the non-metallo-β-lactamase producing resistant strains and the sensitive strains does not implicate differences in the porin proteins in increased resistance to imipenem. These findings are in contrast to those of Piddock and Wise (1987), who described a *B.fragilis* isolate resistant to cefoxitin (not due to β-lactamases) in which a protein of 49 kDa, found in the sensitive NCTC type strain, was absent. They presented this as evidence for cefoxitin resistance due to altered porin proteins. The highly cefoxitin resistant *B.fragilis* R212 (MIC 128 mg/l, See section 3.5) examined in the present study, which produced β-lactamases capable of rapid hydrolysis of cefoxitin (See section 4.5), showed no loss of OM proteins compared to the sensitive strains, despite a high crypticity value.

In this study, the metallo-β-lactamase producing *B.fragilis* isolates surprisingly displayed an additional 40 kDa band that was not present in the sensitive strains or the non-metallo-β-lactamase producers with increased imipenem resistance. It is possible that this band represents β-lactamase, but this could not be confirmed since the β-lactamase enzyme was likely to have been denatured by the SDS-PAGE procedure. However, the majority of reports of the molecular mass of metallo-β-lactamases gave values between 25

kDa and 33 kDa (Hedberg *et al.*, 1992; Payne, 1993), with the exception of Cuchural *et al.* (1986) whose estimate was 44 kDa. Interestingly, Podglajen *et al.* (1992a) also found that the OM protein composition of imipenem metallo-β-lactamase producing resistant mutants differed from that of their sensitive parental strains, and from that of a sensitive reference strain. A decrease in the amount of a 47 kDa OM protein was observed in the imipenem resistant strains, with an increase of a protein with slightly lower molecular weight in the mutant strains. The association between these OM protein changes and antibiotic permeation is unknown.

LPS analysis of *B.fragilis* carried out by Weintraub *et al.* (1985) revealed that most strains have similar, if not identical, LPS of the 'rough' type with one major and one minor band of relatively short polysaccharide chains. In the present study, the *B.fragilis* NCTC control and clinical isolates of normal sensitivity showed three bands, all of low molecular weight. These were identical to the LPS profiles of the *B.fragilis* strains in which decreased susceptibility to imipenem was not enzyme mediated. These results suggest that the LPS composition, or associated hydrophobicity, are not features contributing to the resistance of these strains. Curiously, as with the OM proteins, the metallo-β-lactamase producers differed from the other test strains in their LPS content, as shown by the absence of the higher molecular weight band. This may represent differences in O side chains indicating a different serotype. Different serotypes of *Salmonella* have been shown to exhibit distinct banding patterns on silver-stained gels (Luk and Lindberg, 1991). Similar observations have been made with different serotypes of *Pasteurella multocida* (Rimler, 1990). Another possibility is that these structural differences

indicate that metallo-β-lactamase producing strains of *B.fragilis* belong to a distinct phenotypic sub-group.

Evidence, from the crypticity studies and OM profiles, of a permeability barrier in *B.fragilis* strains that exhibit increased resistance to imipenem in the absence of metallo-β-lactamase activity is, therefore, conflicting. Crypticity studies showed clearly raised crypticity values for two of these strains, but no difference in OM composition was apparent between these and the sensitive strains. It is possible that hydrophobicity is not a feature of imipenem permeation, and examination of gross OM protein content may not have revealed the alteration of porins which was responsible for changes in the efficiency of passage of imipenem.

CHAPTER 7

CHANGES IN PENICILLIN BINDING PROTEINS AS A MECHANISM OF INCREASED RESISTANCE TO IMIPENEM IN *B.FRAGILIS*

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CHANGES IN PENICILLIN BINDING PROTEINS AS A MECHANISM OF INCREASED RESISTANCE TO IMIPENEM IN *B.FRAGILIS*

7:1 Introduction

In addition to β-lactamase activity and reduced permeability, antibiotic resistance may be due to altered binding to essential PBPs. Since PBPs represent the target site for β-lactam antibiotics, changes in these proteins may lead to a decrease in effectiveness of β-lactam agents against the microorganism. Alterations in susceptibility may reflect the number of different PBPs present in the cytoplasmic membrane or modification of the affinity of the PBPs for a particular β-lactam antibiotic. Imipenem resistance has been shown to be associated with modifications of PBPs in *Acinetobacter baumanii*. A comparison of a clinical isolate with an imipenem resistant mutant that was derived from it showed the mutant to have diminished labelling of all but one lower molecular weight PBP (Gehrlein *et al.*, 1991).

Five previous reports have described the number and molecular sizes of PBPs in *B.fragilis* (Botta *et al.*, 1983; Georgopapadakou *et al.*, 1983; Piddock and Wise, 1986; Yotsuji *et al.*, 1988; Wexler and Halebian, 1990). The findings of these studies are conflicting. An investigation of the PBPs of typical, fully sensitive *B.fragilis* strains (*B.fragilis* R116, R135, R192 and NCTC 9344) was therefore carried out to clarify the situation and to provide a basis for comparison with the PBPs of imipenem resistant non-carbapenemase producers.

PBP-associated resistance to cefoxitin attributable to decreased affinity for the PBP1 complex or PBP2 has been reported in laboratory mutants and clinical isolates of *B.fragilis* (Piddock and Wise, 1986; Wexler and Halebian, 1990). Also, resistance to cephalothin and other cephalosporins has been ascribed to reduced binding to PBP3 (Yotsuji *et al.*, 1988). In an attempt to detect any involvement of PBPs in imipenem resistance of *B.fragilis* isolates from this study, the PBPs of strains in which resistance to imipenem was not due to metallo-β-lactamase (*B.fragilis* R208, R212, 0423 and 2013E) were investigated.

Cell membrane proteins of *B.fragilis* strains exposed to ^{3}H-benzylpenicillin were separated on SDS PAGE gels. The radioactive benzylpenicillin, bound to the PBPs, was detected by scintillation counts of discrete gel segments, covering arbitrarily defined molecular weight ranges, or by the more sensitive technique of fluorography (see Section 2.20).

The affinity of various concentrations of imipenem for PBPs of molecular weights greater than or less than 60 kDa from fully sensitive and imipenem resistant *B.fragilis* strains was also measured in competition assays with radiolabelled benzylpenicillin to determine the main regions of imipenem binding.

7:2 Scintillation counts of gel segments

Scintillation counts of proteins in the gel segments were initially measured to ensure the presence of sufficient bound radioactive benzylpenicillin before the lengthy process of fluorography. It became apparent, however, that this method was also useful as a rapid and simple measure of radioactive binding to proteins in gel segments of known molecular weight ranges.

(a) Sensitive B.fragilis strains

Scintillation counts of gel segments containing proteins of molecular weights 100 kDa to 60 kDa and 60 kDa to 20 kDa from experiments in which 5 µl of ^{3}H-benzylpenicillin was used in PBP assays with cell membrane proteins from the four fully sensitive *B.fragilis* strains are given in Table 7.1. The major binding of benzylpenicillin was to proteins of molecular weights from 100 kDa to 60 kDa; between 83% and 91% of the total bound labelled penicillin was present in this gel segment. The proteins of molecular weights between 60 kDa and 20 kDa were associated with a maximum of 17% of the total bound ^{3}H-benzylpenicillin.

To examine these binding sites in more detail, the SDS-PAGE gel resulting from a PBP assay with *B.fragilis* NCTC 9344 was divided up into five segments covering the approximate molecular weight ranges >100 kDa, 100 kDa-75 kDa, 75 kDa-50 kDa, 50 kDa-25 kDa, <25 kDa. The scintillation counts of these segments are shown in Table 7.2. Most of the ^{3}H-benzylpenicillin was associated with the high molecular weight proteins, particularly those between 100 kDa and 75 kDa to which 76% of the labelled benzylpenicillin bound.

A competition assay was performed with imipenem and *B.fragilis* R135 to ascertain the degree and site of imipenem binding to cell membrane proteins of this sensitive strain. A high degree of binding of imipenem was seen with proteins of molecular mass 100 kDa to 60 kDa (Table 7.3). Labelled benzylpenicillin in this segment was reduced generally in proportion to the concentration of imipenem used in the assay, imipenem in the range of 0.015 mg/l to 0.25 mg/l resulting in a decrease of ^{3}H-benzylpenicillin binding to these proteins from 72% to 18% of the total ^{3}H-benzylpenicillin bound in the

Table 7.1 Scintillation counts of gel segments of cell membrane proteins from sensitive *B.fragilis* strains exposed to 5 µl ^{3}H-benzylpenicillin.

Bacterial strains	Scintillation counts^{+} in gel segments containing proteins of mol. wt. (kDa)	
	100-60	60-20
B.fragilis R116	1200(83)	250(17)
B.fragilis R135	1373(91)	141(9)
B.fragilis R192	1200(86)	195(14)
B.fragilis NCTC 9344*	1715(92)	154(8)

* control strain

Figures in brackets represent the percentage of radioactive label detected in each segment of gel.

$^{+}$ counts per minute above the background count

Table 7.2 Scintillation counts of gel segments containing cell membrane proteins from *B.fragilis* NCTC 9344 exposed to 5 μl ^{3}H-benzylpenicillin.

Gel segments containing proteins of mol. wt. (kDa)	Scintillation counts[+]
> 100	51(3)
100-75	1501(76)
75-50	264(14)
50-25	94(5)
< 25	25(2)

Figures in brackets represent the percentage of radioactive label detected in each segment of gel.

[+] counts per minute above the background count

Table 7.3 Scintillation counts of gel segments containing cell membrane proteins from *B.fragilis* R135 in PBP competition assay with imipenem.

Imipenem conc. (mg/l)	Scintillation counts[+] of gel segments containing proteins of mol. wt.[*]: 100-60 kDa	60-20 kDa
0	1417 (96)	63 (4)
0.015	1059 (72)	45 (3)
0.03	562 (38)	39 (3)
0.06	389 (26)	31 (2)
0.12	432 (29)	29 (2)
0.25	263 (18)	59 (4)

[*] figure in brackets show the percentage of total labelled penicillin bound in absence of imipenem

[+] counts per minute above the background count

absence of imipenem. The degree of binding of labelled benzylpenicillin to proteins of molecular weights 60 kDa - 20 kDa was again minor (4% of total bound labelled penicillin). The decrease in bound ^{3}H-benzylpenicillin correlated with the presence of increasing amounts of imipenem in the assay, with the exception of an anomaly with 0.25 mg/l imipenem. The lowest level of binding was seen with 0.12 mg/l imipenem, at which concentration 46% of the binding of ^{3}H-benzylpenicillin, without imipenem, to these segment proteins was observed.

(b) B.fragilis strains in which reduced susceptibility to imipenem was not associated with metallo-β-lactamase

PBP assays in which *B.fragilis* strains were exposed to 5 µl and 15 µl of ^{3}H-benzylpenicillin were carried out to investigate the location of the major penicillin binding sites in the strains exhibiting reduced susceptibility to imipenem and to determine whether the increase in amount of ^{3}H-benzylpenicillin would be reflected in increased binding of radioactive benzylpenicillin.

The scintillation counts obtained with gel segments containing proteins of molecular weights 100 kDa - 60 kDa and 60 kDa - 20 kDa in these experiments are given in Table 7.4. These results were markedly different from those obtained with the sensitive strains. The total counts were reduced on average by a factor of five in experiments with 5 µl ^{3}H-benzylpenicillin (See table 7.1), and were concentrated in the segments containing proteins of molecular weights 60 kDa to 20 kDa.

A threefold increase in the amount of ^{3}H-benzylpenicillin used in the PBP assay resulted in a less than twofold increase in binding to proteins of 60 kDa - 20 kDa for all four test strains and the NCTC

Table 7.4 Scintillation counts of gel segments from PBP assays in which cell membranes of *B.fragilis* strains with non-β-lactamase mediated increased resistance to imipenem were exposed to 5 μl and 15 μl ^{3}H-benzylpenicillin

Bacterial strains	Gel segments containing proteins of mol. wt. (kDa)	Scintillation counts^{+} in assays in which the amount of ^{3}H-benzylpenicillin was: 5μl	15μl
B.fragilis R208	100-60	146	195
	60-20	165	249
B.fragilis R212	100-60	142	191
	60-20	167	242
B.fragilis 0423	100-60	160	155
	60-20	184	244
B.fragilis 2013E	100-60	141	289
	60-20	215	370
B.fragilis NCTC 9344*	100-60	1550	2218
	60-20	135	162

$^{+}$ counts per minute above the background count

* control strain

control. Increase in binding to proteins in the 100 kDa - 60 kDa region was even more modest although a twofold increase was observed with *B.fragilis* 2013E.

Competition by imipenem for the binding of ^{3}H-benzylpenicillin was examined in a resistant *B.fragilis* strain (*B.fragilis* R212). The results of these experiments are given in Table 7.5. The impact of imipenem on the binding of labelled benzylpenicillin was overall less than that of the sensitive strain. The reduction in scintillation counts correlated with an increase in the imipenem concentration present in the PBP assay and, in contast with the sensitive strain (See table 7.3), was generally more apparent in the gel segment containing proteins of molecular weights less than 60 kDa.

7:3 Fluorography

(a) Sensitive B.fragilis strains

The PBPs of the fully sensitive *B.fragilis* strains R116, R135, R192 and NCTC 9344 were resolved by fluorography in experiments in which 5 µl ^{3}H-benzylpenicillin was used. Their molecular weights varied slightly between, but not within, four duplicate experiments (Table 7.6). A typical fluorograph is shown in Fig. 7.1. Three important penicillin binding sites were detected with mean molecular weights of 91 kDa, 80 kDa and 69 kDa (designated PBPs 1, 2 and 3 respectively); these bands were found in all four sensitive strains on all four occasions. In one experiment fainter bands representing two additional PBPs with low molecular weights were detected: *B.fragilis* R135 and NCTC 9344 appeared to possess a 63 kDa protein (designated PBP 4) and *B.fragilis* R135 yielded an additional diffuse band of *ca.* 47 kDa (designated PBP 5).

Table 7.5 Scintillation counts of gel segments containing cell membrane proteins from *B.fragilis* R212 in PBP competition assay with imipenem.

Imipenem conc. (mg/l)	Scintillation counts[+] of gel segments containing proteins of mol. wt.[*]:	
	100-60 kDa	60-20 kDa
0	144(49)	148(51)
0.12	152(52)	134(46)
0.25	108(37)	155(53)
0.5	143(49)	93(32)
1	102(35)	102(35)
2	120(41)	85(29)

[*] figure in brackets show the percentage of total labelled penicillin bound in absence of imipenem

[+] counts per minute above background count

Table 7.6 Molecular weights of PBPs of 4 fully sensitive *B.fragilis* strains (including *B.fragilis* NCTC 9344) determined in 4 separate experiments with 5 µl ^{3}H-benzylpenicillin

	Molecular weight[+] (kDa) in experiment				
PBP	1	2	3	4	mean
1	90	90	90	92	91[x]
2	79	79	79	83	80[x]
3	66	70	69	73	69[x]
4	63[*]	ND	ND	ND	63
5	47[#]	ND	ND	ND	47

ND - Not Detected

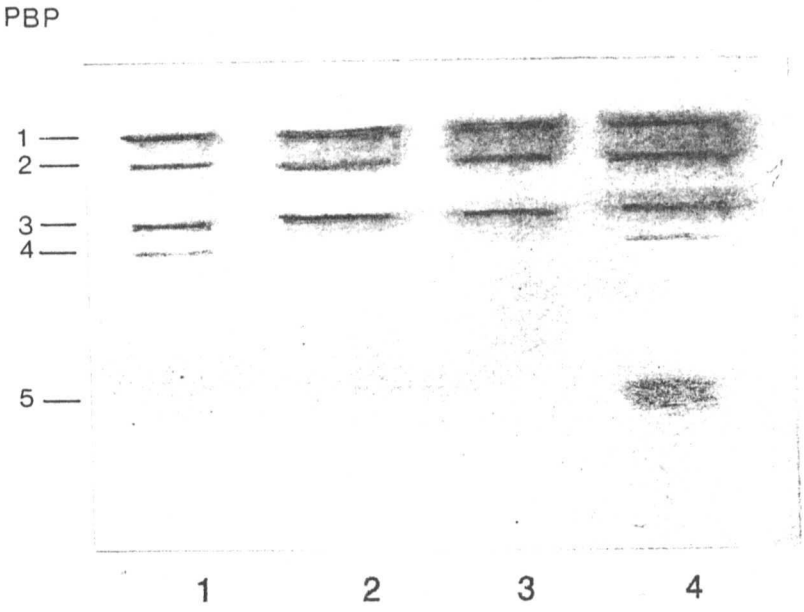
[+] Molecular weights for each PBP determined in each experiment were identical

[x] PBPs 1, 2 and 3 detected in all four test strains on each occation

[*] Detected in *B.fragilis* R135 and NCTC 9344

[#] Detected in *B.fragilis* R135 only as a diffuse band

Fig. 7.1 A typical fluorograph showing PBPs of fully sensitive *B.fragilis* strains



Lanes: 1, *B.fragilis* NCTC 9344; 2, *B.fragilis* R116; 3, *B.fragilis* R192; 4, *B.fragilis* R135

(b) B.fragilis with increased resistance to imipenem which was not associated with metallo-β-lactàmase

No PBPs were seen with *B.fragilis* R208, R212, 0423 and 2013E, in experiments in which 5 µl ^{3}H-benzylpenicillin was used. However, with 15 µl of labelled benzylpenicillin present in the PBP assay, PBPs were visualised as listed in Table 7.7 and illustrated in Fig. 7.2. A PBP of 40 kDa (designated PBP 6), which had not been observed in experiments with fully sensitive strains, was detected in all four resistant isolates but not in the *B.fragilis* NCTC sensitive control. *B.fragilis* 2013E also possessed each of the PBPs found in the sensitive strains, including a 62 kDa and 48 kDa protein. The 48 kDa band was also present in *B.fragilis* 0423, but higher molecular weights bands were not detected in this strain. The 40 kDa band was the only PBP detected in *B.fragilis* R208 and R212 .

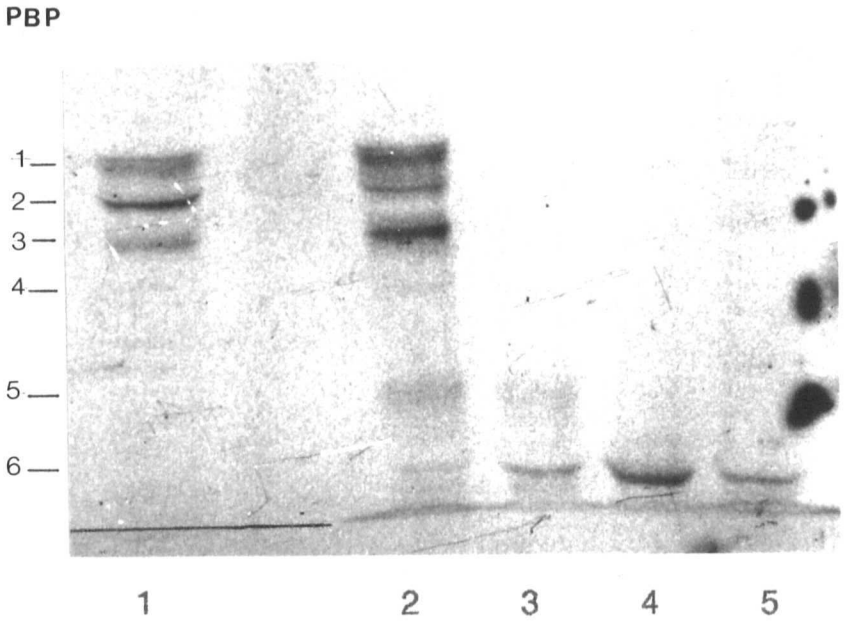
These strains were originally selected on the basis of elevated β-lactamase production. Low penicillinase levels persisted in their cell membrane preparations which were used in PBP assays despite washing five times in buffer (See section 2.20). This is in contrast to the sensitive strains, in which no penicillinase activity was detected in the washed membrane preparations. The hydrolysis of ^{3}H-benzylpenicillin by β-lactamases during the PBP assay may, therefore, have been responsible for the low numbers of PBPs resolved by these strains. Five general methods were considered in order to overcome the presence of β-lactamase in the PBP assays: removal, inactivation, separation or inhibition of the enzyme and addition of excess ^{3}H-benzylpenicillin (Table 7.8). Repeated washing (10 times) of the cell membrane preparations in phosphate buffer failed to remove all penicillinase activity. Complete inactivation of the β-lactamases was not achieved when the cell membrane

Table 7.7 Molecular weights of PBPs of *B.fragilis* strains showing non-β-lactamase mediated resistance to imipenem detected in experiments with 15 μl ^{3}H-benzylpenicillin

		Molecular weight (kDa) of PBP					
Bacterial strain		1	2	3	4	5	6
B.fragilis	R208	ND	ND	ND	ND	ND	40
B.fragilis	R212	ND	ND	ND	ND	ND	40
B.fragilis	0423	ND	ND	ND	ND	48*	40
B.fragilis	2013E	90	79	70	62	48*	40
B.fragilis NCTC 9344+		90	79	70	ND	ND	ND

ND - Not Detected
* diffuse band
+ control strain

Fig. 7.2 Fluorograph showing PBPs of imipenem resistant non-metallo-β-lactamase producing *B.fragilis* strains from experiments with 15 µl ^{3}H-benzylpenicillin



Lanes: 1, *B.fragilis* NCTC 9344 (sensitive control strain); 2, *B.fragilis* 2013E; 3, *B.fragilis* 0423; 4, *B.fragilis* R212; 5, *B.fragilis* R208

Table 7.8 Possible methods to overcome presence of β-lactamases in cell membrane preparation used in PBP assays

Removal
Repeated washing (x10) in phosphate buffer

Inhibition with:
clavulanic acid
sulbactam
p-chloromercuribenzoate
N-ethylmaleinide

Inactivation
temperature 4°C
" 65°C for 10 minutes
sodium azide

Separation
iso-electric focusing
SDS-PAGE
electroelution

Adding excess ^{3}H-benzylpenicillin

preparations were maintained at 4°C or exposed to the enzyme poison, sodium azide (10mM). The β-lactamases were totally inactivated as a result of heating the cell membranes at 65°C for 10 minutes, but these conditions were found to prevent binding of radiolabelled benzylpeniciilin to the PBPs. Physical separation of the β-lactamases from the PBPs was not attempted because of the likelihood of PBP damage by the techniques involved. In the presence of the inhibitors pcmb (1mM) and N-ethylmaleinide (5mM), cell membrane penicillinase activity persisted, whereas with clavulanic acid (5 mg/l) and sulbactam (10 mg/l) the penicillinase reaction showed very weak or negligible activity.

Therefore, in an attempt to resolve the PBPs of these *B.fragilis* strains, the volume of labelled benzylpenicillin used in the PBP assay was further increased to 30 μl and, in other experiments, β-lactamase inhibitors were used. Also, to aid visualisation of weak PBP bands, the exposure time of the radioactive gel with the film at -70°C was increased from three to seven weeks.

With 30 μl ^{3}H-benzylpenicillin present in the PBP assay, three bands were visualised for *B.fragilis* R208 and 0423, corresponding to proteins of molecular weights 76 kDa, 67 kDa and 37 kDa. Results with *B.fragilis* 2013E were similar to those achieved with 15 μl ^{3}H-benzylpenicillin, with six proteins showing of 87 kDa, 76 kDa, 67 kDa, 60 kDa, 45 kDa and 37 kDa. With *B.fragilis* R212, only the 37 kDa protein was observed (Table 7.9, Fig. 7.3).

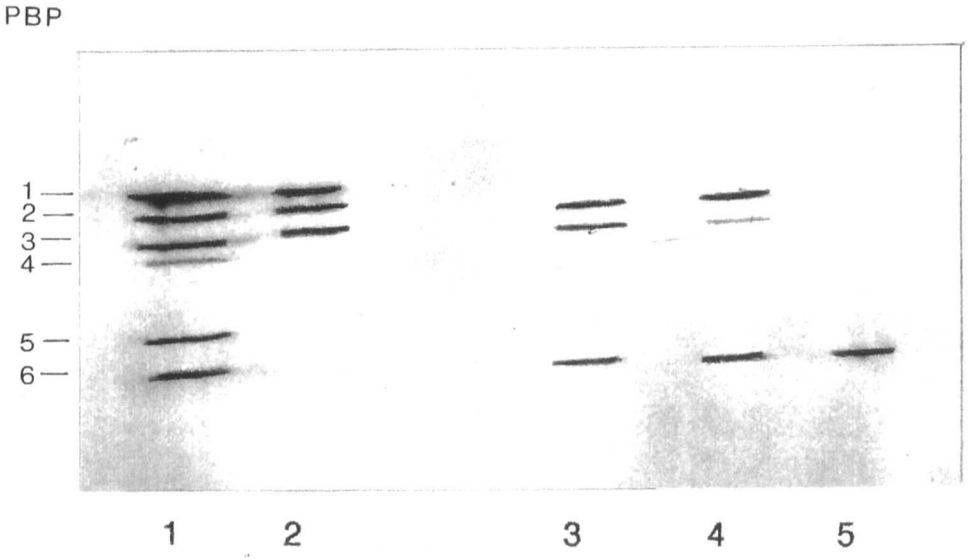
The PBP assays with 10 μl ^{3}H-benzylpenicillin were repeated in the presence of the β-lactamase inhibitors clavulanic acid (5 mg/l) and sulbactam (10 mg/l) (Table 7.10, Fig. 7.4). All four strains now showed PBPs of 88 kDa, 78 kDa and 68 kDa in the presence of

Table 7.9 Molecular weights of PBPs of *B.fragilis* strains showing non-β-lactamase ·mediated resistance to imipenem detected in experiments with 30 μl ^{3}H-benzylpenicillin

Bacterial strain	Molecular weight (kDa) of PBP					
	1	2	3	4	5	6
B.fragilis R208	ND	76	67*	ND	ND	37
B.fragilis R212	ND	ND	ND	ND	ND	37
B.fragilis 0423	ND	76	67	ND	ND	37
B.fragilis 2013E	87	76	67	60*	45	37
B.fragilis+ NCTC 9344	87	76	67	ND	ND	ND

ND not detected
* faint band
+ control strain

Fig. 7.3 Fluorograph showing PBPs of imipenem resistant non-metallo-β-lactamase producing *B.fragilis* strains from experiments with 30 μl ^{3}H-benzylpenicillin



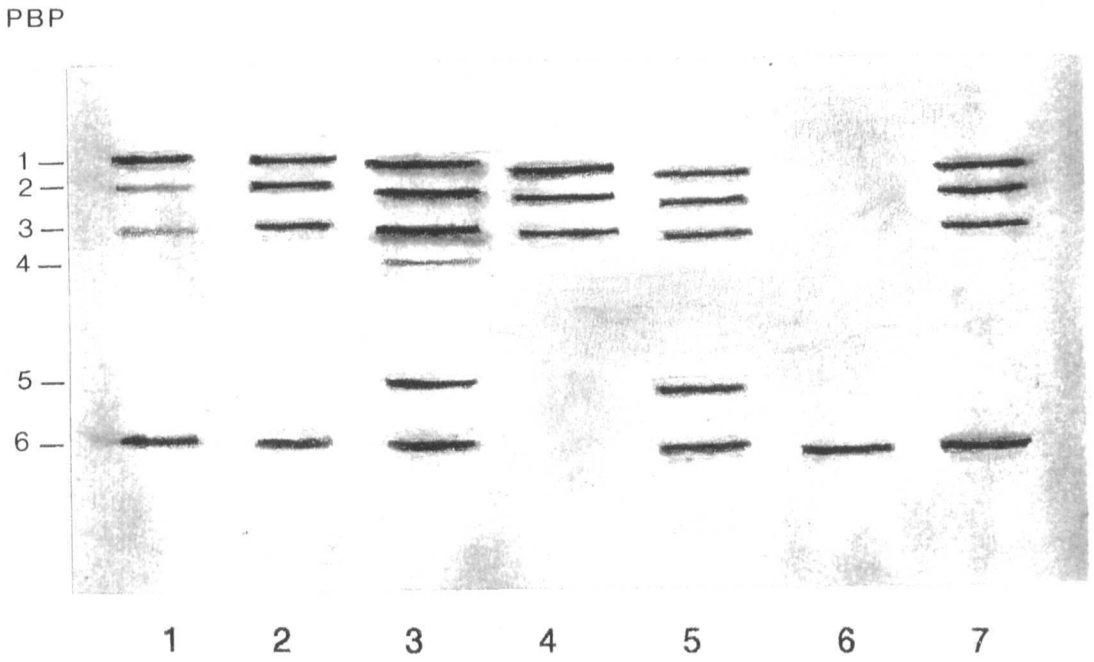
Lanes: 1, *B.fragilis* 2013E; 2, *B.fragilis* NCTC 9344 (sensitive control strain); 3, *B.fragilis* 0423; 4, *B.fragilis* R208; 5, *B.fragilis* R212

Table 7.10 Molecular weights of PBPs of *B.fragilis* strains detected in experiments with 10 µl ^{3}H-benzylpenicillin in the presence of clavulanic acid or sulbactam.

Bacterial strain		Molecular weight (kDa) of PBP					
		1	2	3	4	5	6
B.fragilis R208	CA	88	78	68	ND	ND	37
	SB	88	78	68	ND	ND	37
B.fragilis R212	CA	88	78*	68*	ND	ND	37
	SB	ND	ND	ND	ND	ND	37
B.fragilis 0423	CA	88	78	68	62*	45	37
	SB	88	78	68	ND	45	37
B.fragilis 2013E	CA	88	78	68	ND	ND	ND
	SB	NT	NT	NT	NT	NT	NT
B.fragilis^{+}	CA	88	78	68	ND	ND	ND
NCTC 9344	SB	NT	NT	NT	NT	NT	NT

CA with clavulanic acid (5 mg/l)
SB with sulbactam (10 mg/l)
ND not detected
NT not tested
* faint band
$^{+}$ control strain

Fig. 7.4 Fluorograph showing PBPs of imipenem resistant non-metallo-β-lactamase producing *B.fragilis* strains from experiments carried out in the presence of clavulanic acid and sulbactam



Lanes: 1-4 with clavulanic acid (5 mg/l) 1, *B.fragilis* R212; 2, *B.fragilis* R208; 3, *B.fragilis* 0423; 4, *B.fragilis* 2013E
Lanes 5-7 with sulbactam (10 mg/l) 5, *B.fragilis* 0423; 6, *B.fragilis* R212; 7, *B.fragilis* R208

clavulanic acid, although the latter two bands only appeared faintly in *B.fragilis* R212. These three PBPs, however, were not detected in *B.fragilis* R212 in the presence of sulbactam. *B.fragilis* 0423 was the only strain tested to show bands of 62 kDa and 45 kDa. A 37 kDa PBP was present in all the strains tested with both β-lactamase inhibitors, except for *B.fragilis* 2013E and the NCTC 9344 sensitive control.

7:4 Discussion

The demonstration of PBPs and the assessment of the molecular size depend on the method used to prepare the cell membrane, on the exact method of electrophoresis and on the techniques used to calculate the molecular weights.

In the study, the PBPs of *B.fragilis* showing normal susceptibility to β-lactam agents were revealed by fluorography after preliminary investigation of gel segments. Three important binding sites were apparent, comprising proteins with molecular weight exceeding 60 kDa.

Previous workers have provided conflicting accounts of the number and molecular weights of *B.fragilis* PBPs. Generally five PBPs with molecular weights ranging from 100 kDa to 32 kDa have been described. Botta *et al.* (1983) used ^{14}[C]-cefotaxime to investigate the PBP profile of two strains of *B.fragilis*, and found only three PBPs with molecular weights of 76 kDa, 56 kDa and 53 kDa. The validity of using labelled cefotaxime as a PBP probe was questioned by Piddock and Wise (1983) who emphasised the poor affinity between the antibiotic and PBP 4, 5 and 6 of *E.coli* K-12. They suggested that the low number of PBPs observed by Botta *et al.* (1983) was due to poor binding of cefotaxime and insufficient antibiotic in the assay. Georgopapadakou *et al.* (1983) examined

eight strains of *B.fragilis* and found three PBPs with molecular weights of 100 kDa, 86 kDa and 65 kDa which showed high affinity for imipenem. Occasionally, additional PBPs of 78 kDa and 32 kDa were seen. In 1986, Piddock and Wise investigated the PBPs of *B.fragilis* and three other *Bacteroides* species. In *B.fragilis* they demonstrated a PBP 1 complex with molecular weights ranging from 82 kDa to 78 kDa; two other PBPs of 75 kDa and 70 kDa were also present. Yotsuji *et al.* (1988) described PBPs of molecular weights 94 kDa-88 kDa (PBP 1 complex), 82 kDa (PBP 2) and 72 kDa (PBP 3). Wexler and Halebian (1990) reported similar results for *B.fragilis* ATCC 25285: high molecular weight PBPs of 91 kDa (PBP 1), 83 k Da (PBP 2) and 69 kDa (PBP 3). However, two additional low molecular weight PBPs, of 64 kDa (PBP 4) and 40 kDa (PBP 5) were also reported for this strain in addition to bands of 55 kDa and 33 kDa which were described as 'regions of non-specific binding'. In two clinical isolates of *B.fragilis* examined by these workers, one of which was resistant to cefoxitin, PBPs 2 and 4 could not be detected. The resistant isolate also possessed PBPs of 50 kDa, 39 kDa and 29 kDa. These previous reports are summarised in Table 7.11.

The results of the present study with sensitive *B.fragilis* strains broadly agree with those described for two *B.fragilis* isolates by Yotsuji *et al.* (1988) and *B.fragilis* ATCC 25285 reported by Wexler and Halebian (1990). The PBPs of 91 kDa, 80 kDa and 69 kDa which were found consistently for all four sensitive strains tested correspond closely to the PBPs with molecular weights of PBP 1, 2 and 3 described by these authors. In addition, a PBP of 63 kDa was occasionally detected in two of the four sensitive *B.fragilis* strains examined. This presumably corresponds to Wexler and Halebian's PBP 4 of 64 kDa. The protein of 47 kDa that was seen as a diffuse

Table 7.11 Molecular weights (kDa) of PBPs of *B.fragilis* reported in the literature ·

Botta *et al.* (1983)	Georgopapadakou *et al.* (1983)	Piddock and Wise (1986)	Yotsuji *et al.* (1988)	Wexler and Halebian (1990) +	Wexler and Halebian (1990) S	Wexler and Halebian (1990) R	Present study
	100(1)						
			94(1a)				
			90(1b)	91(1)			91(1)
	86(2)		88(1c)		86(1)		
		82(1a)	82(2)	83(2)			80(2)
		80(1b)				80(1)	
	78[f]	78(1c)					
76(1)		75(2)					
	65(3)	70(3)	72(3)	69(3)	66(3)		69(3)
				64(4)		64(3)	63(4)*
56(2)							
53(3)							
						50(5)	47(5)**
				40(5)		39	40(6)§
	32[f]					29	

The designated PBP are in parentheses

[f] occasionally detected

[+] *B.fragilis* ATCC 25285

S Cefoxitin sensitive isolate

R Cefoxitin resistant isolate

§ found in imipenem resistant strains only

* found in *B.fragilis* R135 and NCTC 9344 only

** found in *B.fragilis* R135 only as a diffuse band

band in *B.fragilis* R135 in one experiment may correspond to the 50 kDa PBP of the cefoxitin resistant isolate examined by Wexler and Halebian (1990).

The molecular sizes determined for the individual PBPs in the study were uniform within experiments for the four sensitive strains tested, although between experiments the value for the same PBP varied by a maximum of 7 kDa. Georgopapadakou *et al.* (1983) and Wexler and Halebian (1990), who stated the molecular weights of PBPs of individual *B.fragilis* strains, showed greater ranges of 14 kDa and 11 kDa respectively. The resolution of constituent bands of PBP 1 reported by some authors (Piddock and Wise, 1986; Yotsuji *et al.*, 1988) was not achieved in this study, possibly due to the size of gel employed.

Scintillation counts of SDS-PAGE gels of membrane proteins from *B.fragilis* NCTC 9344 indicated that the major binding region for benzylpenicillin lay in the part of the gel containing proteins with molecular weight between 100 kDa and 75 kDa, that is PBPs 1 and 2. The low level of binding to proteins of less than 50 kDa proved insufficient to produce bands on the fluorograph.

In an attempt to increase the level of bound radioactivity in order to study the PBPs of the imipenem resistant strains, the volume of ^{3}H-benzylpenicillin in the PBP assay was increased three-fold to 15 µl. In these experiments, a low molecular weight band of 40 kDa was revealed for all four resistant strains, but not for the sensitive control. Wexler and Halebian (1990) found a 40 kDa PBP in a fully sensitive *B.fragilis* ATCC strain and a 39 kDa PBP in a cefoxitin resistant clinical isolate. Scintillation counts of SDS-PAGE gel segments reflected the results obtained by fluorography; the increase

in binding to lower molecular weight proteins (60 kDa-20 kDa) was greater for the resistant strains than for the sensitive *B.fragilis* NCTC control.

These results raise the possibility that the hydrolysed radioactive benzylpenicillin binds, in the form of radioactive fragments, to non-specific binding regions comprising low molecular weight cell membrane proteins of less than 60 kDa which do not exclusively bind intact β-lactam molecules. Wexler and Halebian (1990) described bands from a fluorograph of *B.fragilis* representing proteins below 60 kDa as non-specific binding sites. Low molecular weight PBPs have been described as non-essential and associated with carboxypeptidase and weak penicillinase production in *B.fragilis* and *P.aeruginosa* (Georgopapadakou *et al.* 1983; Livermore, 1987). The low molecular weight (40 kDa) PBP 6 may represent β-lactamase which binds to ^{3}H-benzylpenicillin. Georgopapadakou *et al.* (1983) reported 32 kDa PBPs in three out of eight *B.fragilis* strains studied, including one strain which was resistant to β-lactam antibiotics. Interestingly, binding to this PBP was found to be inhibited by clavulanic acid.

The greatest increase in binding to proteins of molecular weight above 60 kDa of the four resistant strains, as measured by scintillation counts of gel segments and the appearance of PBP bands by fluorography, was seen with *B.fragilis* 2013E. With this strain, the ^{3}H-benzylpenicillin present may have saturated the β-lactamase leaving more of the drug to bind to the major PBPs. However, the specific activity of *B.fragilis* 2013E β-lactamase with nitrocefin as substrate was higher than that of enzymes from the other three resistant test strains (Eley and Greenwood, 1986b; see also section 4.2), although a comparison of penicillinase activities would

be more relevant.

In order to further resolve the PBPs of the non-carbapenemase producing imipenem-resistant strains, attempts were made to prevent penicillinase activity during the PBP assay so that increased amounts of labelled benzylpenicillin were available for binding to target proteins. Other workers have used a variety of ways to try to remove β-lactamases from the cell membranes in PBP assays. Botta *et al.* (1983) suspended the cell membrane preparations in buffer containing p-hydroxy mercuribenzoate (phmb), a β-lactamase inhibitor. There is conflicting evidence whether or not phmb interacts with PBPs. Spratt (1977a) showed that phmb did not alter the PBP profile of *E.coli*. However, Curtis and Strominger (1978) and later Broome-Smith and Spratt (1984) reported interaction between p-chloromercuribenzoate (pcmb), a β-lactamase inhibitor closely related to phmb, and PBP 5 of *E.coli*. The β-lactamases from the four test strains in this study were in any case resistant to inhibition by pcmb (section 4.4; See also Eley and Greenwood, 1986b). Piddock and Wise (1986, 1987) and Yotsuji *et al.* (1988) used cell envelope preparations from bacterial cultures in the late logarithmic phase which, they believed, produced reduced levels of β-lactamases. If, after thorough washing, β-lactamase was still detected, the preparation was discarded. However, substantial β-lactamase activity may be present up to, and sometimes including, the stationary phase of growth (Edwards and Greenwood, 1990) particularly in strains of bacteroides that produce increased amounts of enzyme (Eley and Greenwood, 1985). More recently, Wexler and Halebian (1990) performed PBP assays with cell membrane preparations exhibiting β-lactamase activity in the presence of sulbactam (2 mg/l) to achieve visualization of the PBPs. A solution to this problem of hydrolysis of

the ^{3}H-benzylpenicillin during the PBP assay would have been the use of radioactive imipenem as a direct measure of affinity of the antibiotic. Unfortunately, radio-labelled imipenem is not currently commercially available.

The modifications to the PBP assay method that were chosen to combat the presence of β-lactamases in the cell membrane preparations were: further increasing the concentration of radioactive benzylpenicillin from 15 µl to 30 µl in an attempt to saturate the β-lactamase or including the β-lactamase inhibitors clavulanic acid and sulbactam.

By doubling the volume of ^{3}H-benzylpenicillin used from 15 µl to 30 µl, additional PBP bands were seen with *B.fragilis* R208 and 0423. Both these strains showed 76 kDa and 67 kDa proteins, likely to correspond to the PBP 2 and PBP 3, previously detected with the sensitive strains and *B.fragilis* 2013E. The higher molecular weight protein of 87 kDa (PBP1) was not detected with these two strains. PBP 2 was, therefore, labelled in preference to PBP 1 with *B.fragilis* R208 and 0423, supporting the findings of Piddock and Wise (1986) that most β-lactam compounds, including benzylpenicillin, bind preferentially to PBP 2 in *B.fragilis*. Again, PBP 6 was found in the four resistant strains but not in the sensitive control, possibly reflecting the presence of β-lactamases within the cell membrane of resistant strains during the PBP assay.

In the presence of clavulanic acid, PBPs 1, 2 and 3, corresponding to those found with the sensitive *B.fragilis* strains, were seen in all four resistant strains. PBP 5 (45 kDa) was visible only with *B.fragilis* 0423, which also yielded a faint band of 62 kDa, presumably PBP 4. These findings confirm that the previous failure to

demonstrate binding of ^{3}H-benzylpenicillin with these strains was due to hydrolysis by β-lactamases. PBP 6 has previously been shown to be associated with β-lactamase activity, being consistantly observed in imipenem resistant non-metallo-β-lactamase producing strains which produced elevated amounts of β-lactamase, and not found in sensitive *B.fragilis* strains with low levels of β-lactamase activity. Unexpectedly, PBP 6 was demonstrated in all strains, except *B.fragilis* 2013E, despite the presence of β-lactamase inhibitor. It is tempting to speculate that this low molecular weight PBP is associated with non-β-lactamase mediated imipenem resistance, although Wexler and Halebian (1990) observed a 40 kDa PBP in the sensitive *B.fragilis* ATCC 25285 strain.

In the case of *B.fragilis* R212, PBPs 1, 2 and 3 were absent in tests carried out in the presence of sulbactam, as was PBP 4 of *B.fragilis* 0423. Failure to detect PBPs in the presence of sulbactam may represent the competitive affect of the β-lactamase inhibitor or the activity of β-lactamases despite the presence of sulbactam.

The effect of the β-lactamase inhibitors was more marked for *B.fragilis* 0423 and 2013E than for *B.fragilis* R208 and R212 and corresponds to the sensitivity of the enzymes to the β-lactamase inhibitors as demonstrated by the inhibition studies (section 4.4; See also Eley and Greenwood, 1986b). This was indicated by the appearance of additional PBP bands with *B.fragilis* 0423, and the absence of PBP 6, which appears be associated with β-lactamases, with *B.fragilis* 2013E.

Competition assays with a sensitive *B.fragilis* strain indicated that the greatest binding of imipenem was to the higher molecular weight PBPs of more than 60 kDa. This is in agreement with the findings of Piddock and Wise (1986) who reported that imipenem

binds initially to PBP 3, then to PBP 2, and, at concentrations close to the MIC, to PBP 1. In the imipenem resistant strain, the degree of imipenem binding appeared to be reduced and favoured the low molecular weight proteins. It is possible that this apparent reduced affinity of imipenem, particularly to those PBPs of high molecular weight with lethal functions, may play a part in resistance. However, the situation is complicated by the presence of β-lactamases in the PBP competition assay of resistant strains which governed the amount of ^{3}H-benzylpenicillin available for binding and may themselves be inhibited by imipenem. The possibility that imipenem could act as a β-lactamase inhibitor in the competition assay with the resistant strain *B.fragilis* R212 was supported by the β-lactamase inhibition profile of this strain which indicated susceptibility of the enzyme to imipenem (See section 4.4). Therefore, any reduction in penicillin binding resulting from the competitive action of imipenem may be offset by reduced hydrolysis of ^{3}H-benzylpenicillin due to β-lactamase inhibition by imipenem.

Both sensitive *B.fragilis* and strains with increased resistance to imipenem (in the presence β-lactamase inhibitors) have been shown to possess the three major penicillin binding proteins PBPs 1, 2 and 3. Imipenem showed a high degree of binding to these higher molecular weight PBPs of sensitive strains. The affinity of PBPs of the resistant strains for benzylpenicillin or imipenem was difficult to determine due to the presence of β-lactamases and the competitive action of β-lactamase inhibitors. Low molecular weight PBPs were detected in the imipenem resistant non-carbapenemase producers and in some of the sensitive strains. Some of these PBPs may represent non-specific binding sites. The lowest molecular weight PBP (*ca.* 40 kDa) was only present in the imipenem resistant strains

and was associated with β-lactamase activity or possibly non-enzymic resistance.

CHAPTER 8

DISCUSSION OF RESISTANCE MECHANISMS

CHAPTER 8

DISCUSSION OF RESISTANCE MECHANISMS

Of 108 clinically significant bacteroides isolates collected for this study, 21 produced elevated amounts of β-lactamase as judged by enhanced hydrolysis of nitrocefin. This group of isolates contained all the strains which showed increased resistance to benzylpenicillin, latamoxef and imipenem, and nine of the twelve strains that showed increased resisitance to cefoxitin. This reinforces the importance of β-lactamases in the resistance of bacteroides to β-lactam antibiotics (Nord and Hedberg, 1990) and indicates that the vast majority of strains did not possess unusual permeability barriers or alterations in the target proteins. Clinical isolates of β-lactamase-negative bacteroides, in particular, *B.distasonis*, that were resistant to many β-lactam agents have been reported (Finegold, 1989). Interestingly, two out of the three bacteroides isolated in this study that exhibited increased resistance to cefoxitin and which did not produce elevated β-lactamase levels, were identified as *B.distasonis*.

Bacteroides with increased resistance to the test β-lactam antibiotics were, therefore, largely restricted to the 21 strains which produced raised levels of β-lactamase.

Benzylpenicillin

Fourteen of the 21 strains that exhibited enhanced β-lactamase activity were resistant to benzylpenicillin (MIC ≥32 mg/l). Clavulanic acid (4 mg/l) failed to restore full sensitivity (MIC ≤1 mg/l) in only two of these strains (*B.fragilis* R212 and *B.thetaiotaomicron* R233), although a sixteen-fold and four-fold reduction in MIC of

benzylpenicillin respectively was observed. β-Lactamases from both of these strains were moderately sensitive to clavulanic acid as shown by inhibition of hydroysis of nitrocefin (IC_{50}s 1 μM), although pardoxically the inhibitor (clavulanic acid 4 mg/l, equivalent to 20 μM) failed to prevent rapid and complete hydrolysis of benzylpenicillin in studies with crude cell extracts or whole cells. Three of the 14 benzylpenicillin resistant bacteroides, *B.distasonis* R118, *B.fragilis* R208, and *B.ovatus* R215, appeared susceptible to benzylpenicillin in the presence of clavulanic acid although enzymes from these strains were only moderately sensitive to inhibition by clavulanic acid (IC_{50}s 1, 10 and 1 μM respectively), and slow breakdown of benzylpenicillin by crude cell extracts was observed in the presence of the inhibitor. The remaining isolates with MICs of ≥32 mg/l benzylpenicillin, as expected, possessed enzymes that were fully sensitive to clavulanic acid in inhibitor studies (IC_{50}s <0.1 μM) and failed to hydrolyse benzypenicillin in the presence of the inhibitor.

Cefoxitin

Nine of the 21 isolates that produced high levels of β-lactamase were shown to exhibit increased resistance to cefoxitin (MIC ≥32 mg/l), and these also displayed reduced susceptibility to latamoxef or imipenem or both. Crude cell extracts of seven of these strains were capable of some hydrolysis of cefoxitin, although whole cells from all nine resistant strains reduced the activity of the antibiotic to some extent after 22 hours' incubation. Extracts and whole cells of *B.fragilis* R212 and *B.thetaiotaomicron* R233 completly hydrolysed cefoxitin and this was associated with a high degree of resistance to cefoxitin (MICs ≥128 mg/l). High level resistance to cefoxitin in these strains seems clearly β-lactamase mediated. Crude cell extracts from *B.ovatus* R102, *B.distasonis* R118 and *B.fragilis* R208, which

showed low-level resistance to cefoxitin (MICs 32 mg/l), caused no, or in the case of *B.fragilis* R208, minimal, hydrolysis the antibiotic. With whole cells of these strains, more than 70% of the antibiotic control remained. Results obtained with these strains, which hydrolysed the antibiotic feebly, suggested that other resistance factors may have been operating. The moderate degree of degradation of cefoxitin shown with *B.fragilis* R186, R215, R240 and R251 by crude cell extracts and whole cells was reflected in the relatively modest levels of resistance of these strains (MICs 32-64 mg/l). Interestingly, crude cell extracts of *B.fragilis* R249 showed modest hydrolysis of cefoxitin although the strain was judged sensitive (MIC 16 mg/l cefoxitin).

Latamoxef

Five isolates of bacteroides were resistant to latamoxef (MIC ≥32 mg/l). Four, *B.fragilis* R208 and R212, *B.ovatus* R215 and *B.thetaiotaomicron* R233 were highly resistant (MIC >128 mg/l). Crude cell extracts and whole cells of two of these strains, *B.fragilis* R212 and *B.ovatus* R215, completely hydrolysed latamoxef, clearly implicating β-lactamases in the resistance. In the case of *B.thetaiotaomicron* R233, the efficient hydrolysis achieved by crude extracts was not seen in experiments with whole cells. The discrepancy in these results may have been due to the concentration of the β-lactamases present in the crude cell extracts. With *B.fragilis* R208, only modest hydrolysis of the antibiotic was observed in experiments with crude cell extracts or whole cells, suggesting a role for non-β-lactamase mediated resistance. Also, as with cefoxitin, crude cell extracts of *B.ovatus* R102 failed to degrade latamoxef, yet this strain showed modestly increased resistance (MIC 32 mg/l latamoxef). In experiments with whole cells of this strain only slight decay of the antibiotic was observed. These results again suggest

that non-enzymic mechanisms may be responsible for the decreased susceptibility of this strain.

Curiously, crude cell extracts from *B.fragilis* R186, R240, R249 and R251 completely hydrolysed latamoxef after 20 hours' incubation, yet these strains were found to be sensitive to the drug. Unfortunately, breakdown of latamoxef by whole cells of these strains could not be tested as sub-MIC concentrations were below the detection limit of the microbiological assay system.

Imipenem

No bacteroides strain that was highly resistant to imipenem was encountered in the study. However, isolates for which the MIC of imipenem was 2 mg/l or more, at least 16-times greater than the MICs for 'normal' sensitive *B.fragilis*, were considered to show increased resistance. Seven isolates, five *B.fragilis* together with *B.distasonis* R118 and *B.thetaiotaomicron* R233, showed increased resistance to imipenem by this criterion. In all seven cases the MIC of imipenem was equal to or below the breakpoint of 4-8 mg/l that is usually cited as indicating resistance to imipenem in bacteroides (Finegold, 1989; Phillips *et al.*, 1992). Niether crude extracts nor whole cell preparations of the two non-*B.fragilis* strains markedly hydrolysed imipenem, so that resistance factors other than β-lactamase appeared to be involved in these strains. However, because of constraints of time, mechanisms of increased resistance to imipenem were investigated futher only in isolates of *B.fragilis*.

Imipenem owes its high antibacterial activity to several unique features. It has a compact structure (molecular weight 299 kDa) and is a zwitterion, both of these characteristics normally facilitate rapid penetration across the *E.coli* outer membranes (Yoshimura and

Nikaido, 1985). It is also highly stable to most types of β-lactamases (Neu and Labthavikul, 1982) and has been shown to have high affinity for critical PBPs in Gram negative pathogens (Georgopapadakou *et al.*, 1983; Hashizume *et al.*, 1984).

In order to try to elucidate the resistance mechanisms in *B.fragilis* strains showing reduced susceptibility to imipenem, the five carbapenem resistant isolates were considered together with three additional *B.fragilis* strains that had been found in earlier studies (Eley and Greenwood, 1986b). The characteristics of these strains are summarised in Table 8.1.

Metallo-β-lactamase mediated increased resistance

Rapid hydrolysis of imipenem (and meropenem) was shown by crude cell extracts and whole cell preparations of four of the eight resistant strains: *B.fragilis* R186, R240, R251 and 119. These strains all produced metallo-β-lactamases, and displayed a marked inoculum effect; their increased carbapenem resistance was undoubtedly β-lactamase mediated.

There appeared to be a relationship between the degree of imipenem resistance and the specific imipenemase activities, indicating that higher resistance resulted from greater activity or production of metallo-β-lactamases. Therefore, the relatively low MICs of imipenem of the metallo-β-lactamase producing test strains appeared to be related to the low imipenemase activity of these enzymes. This was typified by two other *B.fragilis* isolates, R249 and 97, which appeared fully sensitive to imipenem in MIC titratitions despite their production of metallo-β-lactamases. Enzymes from these strains exhibited low specific activities and this may explain the lack of resistance with imipenem.

These observations indicate the presence, in the *B.fragilis*

Table 8.1. Summary of characteristics of imipenem resistant *B.fragilis* strains indicating likely resistant mechanisms

Characteristic	*B.fragilis* strain R186	R240	R251	119	R212	0423	R208	2013E
MIC (mg/l)	2	4	2	16	2	2	4	2
β-Lactamase type	1	1	1	1	2	2	3	4
% imipenem remaining after 20 hrs incubation with crude cell extracts	5	<5	<5	<5	90	55	72	83
Inoculum effect	Yes	Yes	Yes	Yes	No	No	Slight	Slight
Reduction of MIC of imipenem by clavulanic acid	Δ	Δ	Δ	Δ	No	No	Yes	Yes
Crypticity factor	1	1	2	3	9	6	1	1
OM changes	Yes	Yes	Yes	Yes	No	No	No	No
Presence of PBPs 1, 2, 3 and 6	NT	NT	NT	NT	Yes	Yes	Yes	Yes
Resistance mechanism	metallo-β-lactamases+				permeability barrier		non-metallo-β-lactamases*	

NT = Not Tested

Δ = clavulanic acid does not inhibit metallo-β-lactamases

\+ = *B.fragilis* R251 and 119 are also possibly associated with a permeability barrier

* = together with other unknown resistance factors

population, of strains which produce metallo-β-lactamases, have low specific carbapenemase activity and show low level carbapenem resistance. In the study, four strains of this type were encountered out of 69 *B.fragilis* isolates (6%), while highly resistant carbapenemase producers were not found. Podglajen *et al.* (1992b) reported, in a French study, that the incidence of *B.fragilis* carrying a 'silent' *cfiA* gene showing low resistance to carbapenems is twice as high as highly carbapenem resistant strains which express the gene. The in vitro one-step mutation to high-level carbapenem resistance of *B.fragilis* strains carrying 'silent' carbapenemase genes, described by Podglajen *et al.* (1992a), highlights the potential for development of carbapenem resistance among *B.fragilis*.

A striking feature of the metallo-β-lactamase producers examined in the study was their distinctive OM protein and LPS composition. This may simply reflect a specific biotype since permeability barriers appeared not to be a major feature as demonstrated by the low crypticity values generally found for these strains (See section 6.2). However, in the case of *B.fragilis* 119, crypticity experiments indicated a partial permeability barrier which may be an additional factor in this strain's moderately high resistance to imipenem. Also, the slightly raised crypticity value shown by *B.fragilis* R251 may be relevant.

Increased resistance not associated with metallo-β-lactamases

B.fragilis strains R208, R212, 0423 and 2013E clearly had imipenem resistance mechanisms that were different to those of the strains previously described. They did not produce metallo-β-lactamases and did not rapidly hydrolyse carbapenems. All four strains produced β-lactamases that caused only a slow loss of imipenem. Paradoxically, these strains exhibited OM and LPS profiles

similar to those of fully sensitive *B.fragilis*.

B.fragilis R212 and 0423 possessed β-lactamases of a similar type, type 2 as defined by this study (See section 4.7). Although these enzymes seemed moderately sensitive to clavulanic acid, the β-lactamase inhibitor had no effect on imipenem susceptibility. Also, no inoculum effect was shown in turbidimetric experiments with these strains. β-Lactamases, therefore, did not appear to play any part in the imipenem resistance. However, crypticity tests indicated that these strains possessed substantial permeability barriers, although this was not reflected in their OM protein and LPS composition. Permeability, therefore, appeared to be a factor in the increased resistance of *B.fragilis* R212 and 0423 to imipenem and other β-lactam antibiotics, but the nature of the permeability change remains obscure.

There was no indication of a permeability barrier with *B.fragilis* R208 and 2013E. Evidence has been presented that *B.fragilis* 2013E is a high producer of normal type 4 β-lactamases, and *B.fragilis* R208 produced elevated amounts of type 3 enzyme (See section 4.7). These two enzyme types display certain similarities. Both *B.fragilis* R208 and 2013E showed a slight inoculum effect and, more importantly, some increase in imipenem sensitivity was seen in the presence of clavulanic acid. These findings suggest that the β-lactamases of *B.fragilis* R208 and 2013E, although incapable of the rapid efficient hydrolysis of imipenem displayed by the metallo-β-lactamases, showed weak carbapenemase activity which may have played a part in the increased imipenem resistance of these strains. Other resistance factors are suggested for these strains by the inability of clavulanic acid to fully restore sensitivity to imipenem. Similar observations were described by Livermore and Yang (1987)

who reported slow hydrolysis of imipenem, mediated by chromosomal non-metallo-β-lactamases in *P.aeruginosa,* which protected the bacteria, although insufficiently to cause clinical resistance. Imipenem resistance in a *B.distasonis* strain has been reported by Hurlbut *et al.* (1990) to be associated with a combination of impermeability and the activity of a non-metallo-β-lactamase susceptible to clavulanic acid. Not surprisingly, the enzyme from this *B.distasonis* strain differed from the β-lactamases of *B.fragilis* 2013E and R208 in that it hydroysed cefoxitin and had a pI value of 6.9.

The possibility exists that lack of binding of imipenem to essential PBPs could result in resistance of these strains. However, it has been shown that, in the presence of β-lactamase inhibitors, the four imipenem resistant strains in which metallo-β-lactamases were not found, possessed the three major PBPs (1, 2 and 3) which were present in sensitive *B.fragilis* isolates. The presence of β-lactamase in the cell membrane of the resistant strains proved to be an obstacle to the assessment of the affinity of imipenem to the PBPs. However, the lowest molecular weight PBP detected, PBP 6, was present in imipenem resistant *B.fragilis* strains and absent from sensitive isolates. Although this PBP appeared to be associated with β-lactamase activity, it was also possibly involved in non-enzymic resistance.

Clinical significance

This study revealed the presence of bacteroides strains with susceptibilities to β-lactam antibiotics below the conventional break-points determined in vitro which possessed a variety of resistance mechanisms. The therapeutic implications of these isolates with reduced susceptibility to β-lactam agents remain unclear and require investigation. Conversion of these strains, particularly those which

produce metallo-β-lactamases, to higher resistance levels during therapy is a possible threat.

The proportion of bacteroides showing resistance or reduced susceptibility to 'β-lactamase stable' antibiotics was low and appeared unchanged over a five year period, with the possible exception of a rise in cefoxitin resistance. High level resistance to latamoxef and cefoxitin (MIC >64 mg/l) appeared marginally more common. Future monitoring is required to detect any changes in the degree resistance of *Bacteroides* spp. to these antibiotics.

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PUBLICATIONS AND PRESENTATIONS RESULTING FROM THE STUDY

Page nos.

Journal of Applied Bacteriology 1990, 69, 421–425 3302/02/90

Effect of growth conditions and storage on the specific activity of β-lactamases of *Bacteroides* spp.

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The influences of growth conditions and cold storage on the specific activity of β-lactamases of four strains of *Bacteroides* spp. was studied. Interbatch variation was observed in extracts prepared in an identical way on separate occasions but less variation was observed in extracts prepared from bacteria grown on Brain Heart Infusion agar supplemented with yeast extract, haemin and menadione, than in similar extracts of bacteria grown in broth or on other solid media. The loss of enzyme activity seen during the stationary phase of growth of some strains in broth was minimal during incubation for 48 h on agar. Storage of enzyme extracts at 4°C was associated with loss of enzyme activity, but activity was retained during storage at −70°C for up to 32 days. Freezing and thawing had little effect on enzyme activity.

Bacteroides spp. are commonly responsible for anaerobic infections in man and are often resistant to β-lactam antibiotics (Dornbusch *et al.* 1975; Sutter & Finegold 1976). An important cause of this resistance is the production of β-lactamases (Anderson & Sykes 1973; Britz & Wilkinson 1978; Del Bene & Farrar 1973), for the characterization of which quantitative determinations have been extensively used (Eley & Greenwood 1986a, b; Simpson *et al.* 1982).

Production of β-lactamase by some strains of *Bacteroides* spp. is known to vary throughout the growth cycle (Darland & Birnbaum 1977; Eley & Greenwood 1985). Moreover, medium composition appears to affect β-lactamase production, making comparisons between different studies difficult (Olsson *et al.* 1976; Simpson *et al.* 1982).

We have investigated the specific activities of β-lactamases produced by selected strains of *Bacteroides* spp. under various conditions of growth and have explored the effect of storage conditions on the stability of the enzymes in crude extracts.

Materials and Methods

BACTERIAL STRAINS

Four representatives of the *Bacteroides fragilis* group were studied: *B. fragilis* 2013E, *B. thetaiotaomicron* 0456 and *B. distasonis* R939 were used in an earlier study of the effect of growth phase on β-lactamase production (Eley & Greenwood 1985); *B. ovatus* RE2 was a fresh clinical strain obtained in Nottingham Public Health Laboratory.

EXTRACTION OF β-LACTAMASES

Bacteria were harvested from solid media with a cotton wool swab, or from broth by centrifugation. The organisms were suspended in 4 ml of phosphate-buffered saline (0·02 mol/l; pH 7) in a beaker of iced water. The bacterial suspensions were then subjected to six 30 s bursts of sonication under ice in a Soniprep 150 ultrasonicator (MSE Scientific Instruments). The extracts were centrifuged at 4000 rev/min for 30

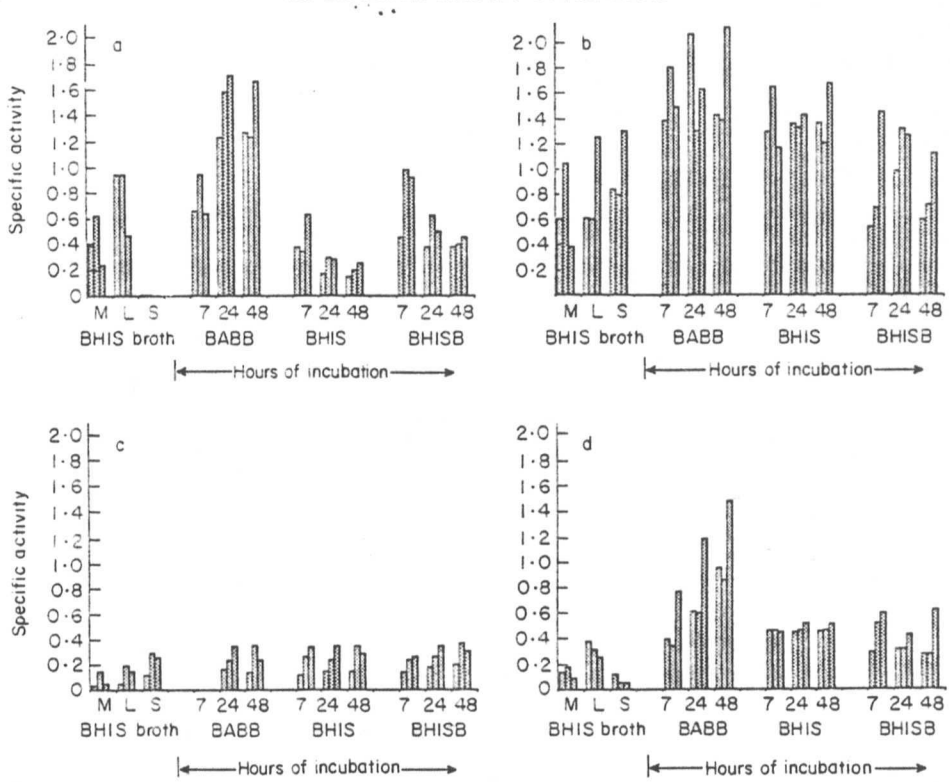

Fig. 1. Specific activities (μm nitrocefin hydrolysed/min/mg protein) of β-lactamases derived from (a) *Bacteroides fragilis* 2013E; (b) *B. thetaiotaomicron* 0456; (c) *B. distasonis* R939; (d) *B. ovatus* RE2 harvested from supplemented Brain Heart Infusion broth and agar (BHIS broth and BHIS), BHIS agar with 7% horse blood (BHISB) and Blood Agar Base with 7% horse blood (BABB) after various times of incubation. Results shown are from three independent experiments. M, mid-logarithmic phase; L, late logarithmic phase; S, stationary phase of growth.

min at 4°C (Coolspin; MSE). Each supernatant fluid (crude extract) was divided into two portions and stored at −70°C for not more than 14 days.

DETERMINATION OF SPECIFIC ACTIVITY

The method used was that of O'Callaghan *et al.* (1972). The rate of hydrolysis of 1·9 ml of nitrocefin (51·6 mg/l solution in phosphate buffer at pH 7) by 0·1 ml of crude enzyme extract was measured over 3 min at 37°C and 482 nm in a spectrophotometer (UV-160A; Shimadzu). Extracts achieving maximum hydrolysis in under 3 min were diluted appropriately in phospate buffer and retested.

The total soluble protein content of the crude extracts was measured with the Sigma Protein Assay kit.

Specific activity was calculated according to the formula:

$$\frac{\Delta \text{O.D./min}}{\text{total } \Delta \text{O.D.}} \times \frac{N}{\text{protein content (mg/ml)}} \times \text{enzyme dilution}$$

where $N = \mu$moles nitrocefin present in the cuvette.

EFFECT OF GROWTH CONDITIONS

The test strains were grown at 37°C in Brain Heart Infusion broth (Oxoid) supplemented with yeast extract 5 g/l, haemin 5 mg/l and menadione 1 mg/l (BHIS) in the anaerobic turbidimeter described by O'Grady & Eley (1983). Bacteria were harvested during the middle and late logarithmic (pre-stationary)

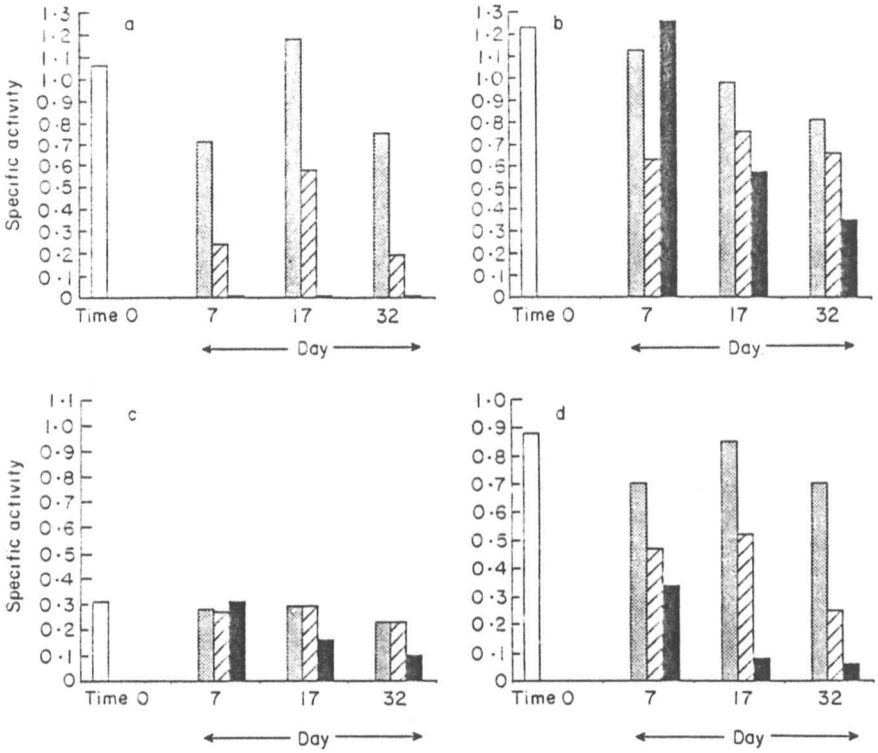

Fig. 2. Specific activities (μm nitrocefin hydrolysed/min/mg protein) of β-lactamases from (a) *Bacteroides fragilis* 2013E; (b) *B. thetaiotaomicron* 0456; (c) *B. distasonis* R939; (d) *B. ovatus* RE2 before and after various cold storage regimens. ▨, −70°C; ▨, −20°C; ■, +4°C.

phases of growth and after 24 h of incubation (stationary phase).

In experiments with solid media three types were used: BHIS solidified with 1% agar (BHIS agar), BHIS agar with 7% horse blood (BHISB) and Blood Agar Base (Oxoid) with 7% horse blood (BABB). All media were pre-reduced overnight in an anaerobic cabinet. Specific activity was determined on extracts of bacteria harvested after incubation for 7, 24 and 48 h in an anaerobic cabinet at 37°C.

Growth and extraction procedures were carried out on three separate occasions for each condition of growth.

To assess intrabatch reproducibility, one extract of each strain was tested for specific activity three times on the same day.

EFFECT OF STORAGE

The specific activities of extracts of the test strains grown on BABB were determined immediately after preparation of the crude extract. Each extract was then divided into nine portions and stored in batches of three at 4°C, −20°C and −70°C. Specific activity was determined after 7, 17 and 32 days in each storage condition.

EFFECT OF FREEZING AND THAWING

Further crude extracts were made of strains *B. fragilis* 2013E and *B. thetaiotaomicron* 0456 grown in BHIS. The specific activity of these extracts was immediately determined. Portions of the extracts were then frozen (at −20°C and −70°C) and thawed three times over a period of 4 h and the specific activities measured on each occasion.

Results

INTRABATCH REPRODUCIBILITY

The maximum deviation from the mean of specific activity values of one extract analysed three times on the same day was 10%.

EFFECT OF GROWTH CONDITIONS

Specific activities of β-lactamases derived from bacteria harvested from broth or three types of solid medium are given in Fig. 1, in which the results of three separate experiments are shown. Specific activities of β-lactamases of *B. fragilis* 2013E and *B. ovatus* RE2 increased during the late logarithmic (pre-stationary) phase of growth in broth and declined dramatically during the stationary phase. Such loss of activity on prolonged incubation was absent or much less marked during growth of these strains on solid media for up to 48 h.

Specific activities of the β-lactamases of *B. thetaiotaomicron* 0456 and *B. distasonis* R939 were vastly different. However, the activities of both enzymes were retained in the stationary phase of growth in broth and during incubation for 48 h on solid media.

Growth on BABB yielded greater specific activity than that observed after growth on BHIS or BHISB agars. This was particularly marked for *B. fragilis* 2013E and *B. ovatus* RE2. Considerable variation in specific activity was observed among samples processed in an identical manner on three separate occasions. In some, values recorded varied by a factor of two or three.

EFFECT OF STORAGE

The impact of cold storage on the β-lactamase activity of extracts of the test strains is shown in Fig. 2. Stability of the enzyme varied somewhat from strain to strain, but marked loss of activity occurred during storage at 4°C with all strains. For three of the four extracts, enzyme activity was retained more completely during storage at −70°C than at −20°C.

EFFECT OF FREEZING AND THAWING

Freezing at −70°C and subsequent thawing had little or no effect on β-lactamase activity, but in experiments in which freezing was carried out at −20°C, a small loss of activity was observed, particularly with *B. fragilis* 2013E.

Discussion

Comparison of the results of studies of β-lactamases is often difficult, because of the possibility that methodological differences might influence the findings. This is a particular problem in studies of *Bacteroides* spp., in some strains of which β-lactamase production is known to vary with the phase of growth (Darland & Birnbaum, 1977; Eley & Greenwood 1985). In this study we have attempted to define the optimum conditions of growth and storage for the characterization of β-lactamases of species of *Bacteroides* within the *B. fragilis* group. To this end we have evaluated the interbatch and intrabatch reproducibility of assays of specific activity and examined the effects of storage and freezing and thawing on enzyme activity.

Not surprisingly, intrabatch reproducibility proved to be very good (±10%), but the interbatch variation observed was much greater. Thus, when specific activity was measured on samples prepared in an identical fashion on three different occasions, twofold differences in specific activity were commonly found and larger differences were occasionally encountered. Such biological variation in enzyme activity appeared somewhat less marked in samples prepared from bacteria grown on BHIS agar for 24 or 48 h.

In general, the highest yield of β-lactamase activity was obtained on BABB. Whether this effect is attributable to the presence of the horse blood is doubtful, since such a stimulatory effect was not uniformly achieved by addition of horse blood to BHIS agar.

Overall, these results suggest that BHIS agar (without blood) provides a reliably reproducible growth medium for β-lactamase studies of *Bacteroides* spp. Growth on agar seems preferable to that in broth since this obviates, or much reduces the influence of growth phase on enzyme production and therefore renders the time of harvest much less critical. BHIS also has the virtue of providing luxuriant growth of morphologically normal cells of *Bacteroides* spp. (Eley *et al.* 1985). Some other commonly used anaerobic media, such as Schaedler broth, Wilkins-Chalgren broth and thioglycollate broth are less satisfactory in this regard (Eley *et al.* 1985) and were, for this reason, not tested in this study.

Investigation of the stability of β-lactamases during storage revealed considerable loss of activity when extracts were held at 4°C, but almost complete retention of activity during storage at −70°C for up to 32 days. There was

little evidence of loss of activity by repeated freezing and thawing.

Loss of activity at 4°C was greatest in those strains in which loss of β-lactamase activity was observed during the stationary phase of growth in broth. Loss of activity is without doubt associated with proteolytic breakdown of preformed enzyme, an effect which evidently continues during storage at 4°C.

We have previously suggested (Eley & Greenwood 1986a,b) that it might be useful to categorize the β-lactamases of clinical strains of *Bacteroides* spp. on the basis of their specific activities. The present results highlight the necessity to define the conditions of growth and storage if specific activity is to be used in this way.

On the basis of the present findings, we recommend that BHIS agar should be used for the growth of *Bacteroides* spp. and that sonicated extracts, prepared according to a standardized protocol, should be stored at −70°C for not longer than 3 weeks before the estimation of specific activity or use in other enzyme studies.

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Extended abstract

An investigation of β-lactamase-mediated imipenem resistance in clinical isolates of *Bacteroides* spp.

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Imipenem is used in the treatment of anaerobic infections and has excellent antimicrobial activity which is due, in part, to β-lactamase stability. We have determined the degree of resistance to imipenem amongst clinically significant bacteroides isolates. The role of β-lactamases in this resistance was studied by examining imipenem stability to crude enzyme extracts and whole cells, and by investigating the affect of clavulanic acid, a β-lactamase inhibitor, on imipenem activity.

Of 116 clinical bacteroides isolates, seven (6%) were found to be imipenem resistant (MIC >2mg/l). These strains comprised 5 *B.fragilis* (R186, R208, R212, R240, R251), 1 *B.distasonis* (R118) and 1 *B.thetaiotaomicron* (R233). MICs were determined in supplemented Brain Heart Infusion (B.H.I.) agar with 48 hours of incubation.

β-Lactamase activity was investigated by exposing crude

enzyme extracts, obtained by sonication of bacterial cells, to imipenem (50mg/l). The mixture was incubated at 37°C and the residual imipenem assayed by HPLC after 2, 4 and 22 hours. The β-lactamase activity of whole cells was examined using strains grown in supplemented B.H.I. broth containing imipenem (0.25mg/l). After 22 hours of incubation, the residual imipenem was measured by microbiological assay. In addition, the MIC and the breakdown of imipenem by whole cells were tested in the presence of clavulanic acid for those strains whose β-lactamases were known to be affected by this inhibitor.

Enzyme extracts from three *B.fragilis* strains (R186, R240 and R251) were capable of rapid hydrolysis of imipenem. With whole cells, imipenem was detected after incubation with only one strain, *B.fragilis* R212. In previous experiments with penicillin, the ability of clavulanic acid to inhibit these β-lactamases was observed with only two strains, *B.fragilis* R208 and *B.distasonis* R118. In the presence of clavulanic acid, sensitivity to imipenem was restored for both these strains and residual imipenem was detected after incubation with whole cells.

The results suggest that β-lactamases were a factor in the resistance to imipenem for six of the seven bacteroides strains tested. These six strains appeared to break down imipenem in growing cultures, although only three strains demonstrated degradation with crude enzyme extracts. This difference may be due to alteration of the enzymes during the extraction process or to the different imipenem concentrations used in the two experiments. The non-β-

lactamase-mediated resistance of the one strain is presumably associated with low permeability or lack of affinity to the P.B.P.s.

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An investigation of β-lactamases from clinical isolates of *Bacteroides* species

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Summary. Among a group of 116 clinically significant isolates of *Bacteroides* spp., 24 exhibited β-lactamase activity greater than the basal level characteristic of most *Bacteroides* strains. Investigation of specific enzyme activity, iso-electric point and enzyme inhibition profiles revealed that the β-lactamases involved could be divided into four groups, some showing similarity to those described in previous studies. Seven of the enzymes were able to hydrolyse cefoxitin, latamoxef or imipenem, and eight enzymes degraded penicillin in the presence of clavulanic acid. Five strains showed reduced susceptibility to cefoxitin, latamoxef or imipenem which was not associated with β-lactamase activity.

Introduction

The production of β-lactamase is the major factor in the resistance of *Bacteroides* spp. to β-lactam antibiotics. Most *Bacteroides* strains exhibit low-level chromosomally mediated β-lactamase activity that affects benzylpenicillin and many other penicillins, although the enzyme preferentially hydrolyses cephalosporins.[1] A substantial number of clinical isolates show raised levels of β-lactamase activity.[2–4] Some of these enzymes inactivate β-lactamase-stable compounds, including cefoxitin, latamoxef and imipenem, and are insusceptible to inhibition by β-lactamase inhibitors, such as clavulanic acid or sulbactam.[4–8]

Attempts have been made to classify the β-lactamases produced by *Bacteroides* spp. according to their substrate profiles, susceptibility to various enzyme inhibitors, iso-electric point, specific enzyme activity and rate of hydrolysis of susceptible substrates.[3, 7] In a previous communication,[4] we used such methods to examine the β-lactamases of a group of *Bacteroides* strains isolated from clinical specimens in Nottingham, and suggested that those strains exhibiting enhanced levels of enzyme activity could be grouped according to their specific cephalosporinase activity.

We have now examined a further group of clinical isolates to assess the prevalence of the various enzyme types and to try to shed further light on the characterisation of these enzymes.

Materials and methods

Bacterial strains

One hundred and sixteen clinically significant isolates of *Bacteroides* spp. were collected randomly from specimens submitted to Nottingham Public Health Laboratory and maintained in skimmed milk at −70°C. They were identified by carbohydrate fermentation tests and, if these tests yielded equivocal results, by the ATB 32A system (API Products, Basingstoke, Hants). *B. fragilis* NCTC 9344 was used as a control strain.

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Antibiotics

Solutions of benzylpenicillin (Glaxo Laboratories Ltd), clavulanic acid (Beecham Research Laboratories Ltd), cefoxitin (Merck, Sharp and Dohme Ltd) and latamoxef (Eli Lilly and Co. Ltd) were freshly prepared as required in sterile distilled water. Appropriate concentrations of imipenem (Merck, Sharp and Dohme Ltd) were dissolved in 0·01M phosphate buffer, pH 7·0, according to the manufacturer's instructions.

Antibiotic titrations

Minimum inhibitory concentrations (MICs) were determined by the agar incorporation method. Antibiotics in suitable two-fold serial concentrations were incorporated in Brain Heart Infusion Agar supplemented with yeast extract 5 g/L, haemin 5 mg/L, menadione 1 mg/L and lysed horse blood 5%. An inoculum of *c.* 10^6 organisms was delivered on to the agar surface with a multipoint inoculator (Denley Instruments, Billingshurst, Sussex). The lowest antibiotic concentration that completely inhibited growth after incubation for 48 h at 37°C, in an anaerobic cabinet, was taken as the MIC.

Semi-quantification of β-lactamase in intact bacteria

Penicillinase. The method of Escamilla[9] was used. In this test, a pH change occurring as a result of

hydrolysis of benzylpenicillin is assessed by a colour change in a phenol red indicator.

Cephalosporinase. The hydrolysis of nitrocefin (250 mg/L), which results in a colour change from yellow to red, was used as described by Aldridge *et al.*[10]

In both tests, several colonies from overnight cultures of organisms grown on supplemented Brain-Heart infusion agar were used and the results were assessed visually over a 1-h period.

Preparation of crude β-lactamase extracts

Bacterial growth was harvested from Blood Agar plate cultures (Blood Agar Base, Oxoid, with horse blood 7%) after incubation for 48 h in an anaerobic cabinet. The bacteria were suspended in a tube containing 4 ml of phosphate buffer (0·02 M; pH 7·0) in a beaker of iced water. The bacterial suspensions were then subjected to six 30-s bursts of sonication, on ice, in a Soniprep 150 ultrasonicator (MSE Scientific Instruments, Crawley, Sussex). The extracts were centrifuged at 4000 rpm for 30 min at 4°C (Coolspin; MSE) and the supernate (crude extract) was divided into four portions and stored at −70°C.[11]

Determination of specific activity

The method used was based on that of O'Callaghan *et al.*[12] The rate of hydrolysis of nitrocefin (1·9 ml of a 51·6 mg/L solution in phosphate buffer, pH 7·0) by 0·1 ml of crude enzyme extract was measured over 3 min at 37°C and 482 nm in a spectrophotometer (UV-160A; Shimadzu, Kyoto, Japan). Extracts achieving maximum hydrolysis in under 3 min were diluted appropriately in phosphate buffer and re-tested. The total soluble protein content of the crude extracts was measured by the Sigma Protein Assay Kit. Specific enzyme activity was calculated as the amount (μmol) of nitrocefin hydrolysed/min/mg of protein.

Iso-electric focusing

Iso-electric focusing of crude β-lactamase extracts was done on cellulose acetate membranes as previously described.[13]

Inhibition profiles

Enzyme inhibition analysis was performed in a centrifugal fast analyser (Centrifichem 400; Union Carbide, Terrytown, New York, USA) by following the colour change induced in nitrocefin in the presence of serial 10-fold concentrations (range 0·1–100 μM) of imipenem, latamoxef, cefoxitin, clavulanic acid, sulbactam (Pfizer Ltd), cloxacillin (Beecham Research Laboratories Ltd.) and *p*-chloromercuribenzoate (pCMB) (Sigma) as described by Eley and Greenwood.[7] The activities of the crude β-lactamase extracts were standardised before testing by appropriate dilution with 0·02 M phosphate buffer.

High pressure liquid chromatography (HPLC)

The kinetics of hydrolysis of latamoxef, cefoxitin, imipenem and the combination of benzylpenicillin and clavulanic acid (4 mg/L) was investigated by HPLC; 0·2 ml of enzyme extract, concentrated five times by freeze drying, was mixed with 0·8 ml of antibiotic solution to give a final concentration of 50 mg/L and incubated at 37°C. Samples were removed for HPLC analysis at 0, 2, 4 and 20 h. Antibiotic controls, in which phosphate buffer was substituted for enzyme extract, were examined in parallel. A Hypersil 5ODS column (HPLC Technology, Macclesfield, Cheshire) was used. The mobile phase for analysis of benzylpenicillin and cefoxitin was methanol:water 40:60 with detection at wavelengths of 227 nm and 235nm, respectively. For latamoxef, methanol:water 25:75 was used with detection at 250 nm, and for imipenem, methanol:water 20:80 with detection at 300 nm. Phosphoric acid 1% was included as a mobile phase modifier throughout these experiments.

Results

Identification of isolates

The 116 isolates were identified as: *B. fragilis* (69), *B. distasonis* (16), *B. ovatus* (13), *B. thetaiotaomicron* (6), *B. melaninogenicus* (4), *B. vulgatus* (4), *B. bivius* (3) and *B. buccae* (1).

Semi-quantification of enzyme activity

Of the 116 isolates, 28 failed to induce a colour change in nitrocefin within 1 h, 64 produced a weak reaction, and 24 caused a complete colour change. The latter strains (14 *B. fragilis*, 5 *B. ovatus*, 2 *B. bivius*, 1 *B. thetaiotaomicron*, 1 *B. distasonis* and 1 *B. buccae*) were selected for further characterisation of their β-lactamases. Fifteen of the 24 strains that produced enhanced levels of cephalosporinase also gave positive reactions in the Escamilla test for penicillinase.

Antibiotic titrations

MICs of the antibiotics for all 116 test strains are shown in table I. Imipenem and the combination of benzylpenicillin with clavulanic acid were more active than cefoxitin, latamoxef or benzylpenicillin alone. Eleven of the 24 strains with raised β-lactamase levels exhibited increased resistance to benzylpenicillin (MIC > 32 mg/L). Clavulanic acid 4 mg/L reduced the MIC of benzylpenicillin more than two-fold with all but three of the 24 test strains; in all but six cases the susceptibility to benzylpenicillin was reduced to

Table I. Susceptibilities of *Bacteroides* strains to selected β-lactam antibiotics

Antibiotic	Minimum inhibitory concentration (mg/L)		
	Range	MIC50	MIC90
Benzylpenicillin	0·5–>64	16	32
Benzylpenicillin + clavulanic acid	0·06–16	0·25	2
Cefoxitin	0·25–>128	16	32
Latamoxef	0·25–>128	1	16
Imipenem	0·03–4	0·5	2

MIC50 and MIC90, concentrations inhibiting 50% and 90% of strains, respectively.

<2 mg/L in the presence of clavulanic acid 4 mg/L (table II). The strains displayed a wide range of susceptibilities to the other test antibiotics. Four strains, *B. fragilis* R212 and R208, *B. ovatus* R215 and *B. thetaiotaomicron* R233 exhibited markedly reduced susceptibility to cefoxitin, latamoxef and imipenem.

Specific enzyme activity

Specific cephalosporinase activity (μmol of nitrocefin degraded/min/mg of protein) of the crude enzyme extracts ranged from 0·01 to 1·33 (table III). All the strains for which the specific activity was ≥0·1 exhibited high penicillinase activity as determined by the whole-cell semi-quantitative test, whereas many of the β-lactamases with lower specific activity lacked penicillinase activity.

Iso-electric focusing

Crude extracts of *B. fragilis* isolates yielded a single iso-electric point between 4·9 and 5·3 (table III). No band was detected by iso-electric focusing of extracts of three other *B. fragilis* strains (R240, R249, R251) despite repeated testing. Enzymes from the five *B. ovatus* strains displayed a wide range of pI values (4·5–6·7). The two *B. bivius* strains displayed high pI values (>6·0).

Inhibition profiles

Only five of the 24 strains (*B. fragilis* R106, R216 and R226, *B. ovatus* R111 and R112) produced β-lactamases that were at least partially susceptible to all the inhibitors tested; a further seven were susceptible to all but pCMB (table IV). The concentrations of inhibitors required to achieve inhibition of the individual enzymes varied considerably. The β-lactamase from *B. fragilis* R186 was not inhibited by any of the agents tested and three *B. fragilis* strains (R240, R249 and R251) displayed very low levels of inhibition (table IV). There were only three profiles common to more than one strain: those shown by *B. fragilis* R130 and R152; *B. buccae* R147 and *B. bivius* R160; and *B. ovatus* R137 and *B. fragilis* R141.

Table II. Antibiotic susceptibility (MIC mg/L) of 24 strains of *Bacteroides* that exhibited increased β-lactamase activity

Bacterial strain	Minimum inhibitory concentration (mg/L)				
	benzyl penicillin	benzylpenicillin + clavulanic acid	cefoxitin	latamoxef	imipenem
B. fragilis R106	32	0·5	16	1	0·5
B. fragilis R130	32	0·5	8	1	0·25
B. fragilis R249	4	2	16	0·5	1
B. fragilis R251	16	2	32	4	2
B. fragilis R186	8	4	32	1	2
B. fragilis R240	8	4	32	1	4
B. buccae R147	16	<0·12	<0·5	0·5	<0·03
B. ovatus R137	16	0·5	8	1	0·5
B. ovatus R111	16	0·25	4	1	0·5
B. bivius R160	4	<0·12	4	4	0·06
B. distasonis R118	32	1	32	16	2
B. fragilis R208	>64	1	32	>128	4
B. fragilis R141	16	0·25	16	0·5	0·06
B. bivius R143	16	0·25	4	4	0·03
B. fragilis R126	>64	1	16	16	0·5
B. ovatus R215	>64	0·5	64	>128	1
B. fragilis R152	>64	<0·12	8	8	0·12
B. ovatus R112	>64	0·25	16	16	0·5
B. fragilis R226	>64	0·5	8	16	1
B. fragilis R216	>64	1	8	8	1
B. ovatus R102	>64	1	32	32	0·5
B. fragilis R212	>64	4	128	>128	2
B. fragilis R134	>64	0·25	16	16	0·25
B. thetaiotaomicron R233	>64	16	>128	>128	4
B. fragilis NCTC9344*	16	0·25	8	0·25	0·12

* Data from reference 7, included for comparison.

Table III. Characterisation of β-lactamases from 24 strains of *Bacteroides* that exhibited increased β-lactamase activity

Bacterial strain	β-lactamase activity		Specific activity*	Iso-electric point
	cephalosporinase	penicillinase		
B. fragilis R106	+ + +	−	0·01	5·3
B. fragilis R130	+ +	−	0·01	5·0
B. fragilis R249	+ +	−	0·01	ND
B. fragilis R251	−	+ +	0·01	ND
B. fragilis R186	+ + +	−	0·02	4·9
B. fragilis R240	+ +	+ +	0·02	ND
B. buccae R147	+ +	+ +	0·03	5.2
B. ovatus R137	+ +	+ +	0·03	4·5
B. ovatus R111	+ + +	+	0·05	4·8
B. bivius R160	+ + +	−	0·05	6·1
B. distasonis R118	+ +	+	0·07	4·6, 5·0
B. fragilis R208	+ +	−	0·08	5·0
B. fragilis R141	+ +	−	0·09	5·0
B. bivius R143	+ + +	+ + +	0·10	6·4
B. fragilis R126	+ + +	+ + +	0·15	5·0
B. ovatus R215	+ +	+ + +	0·16	5·0, 6·7
B. fragilis R152	+ + +	+ + +	0·21	5·2
B. ovatus R112	+ + +	+ + +	0·44	5·1
B. fragilis R226	+ + +	+ + +	0·68	4·9
B. fragilis R216	+ + +	+ + +	0·71	4·9
B. ovatus R102	+ + +	+ + +	0·87	5·0
B. fragilis R212	+ + +	+ + +	0·88	5·1
B. fragilis R134	+ + +	+ + +	0·93	4·9
B. thetaiotaomicron R233	+ + +	+ + +	1·33	4·6
B. fragilis NCTC9344	±	−	0·03	5·1

+−+ + +, arbitrary levels of enzyme activity assessed visually according to the speed and completeness of the colour change. Isolates that exhibited basal levels of cephalosporinase activity, scored as ± after 1h, are omitted from this table (results for *B. fragilis* NCTC 9344[7] included for comparison).
* Nitrocefin μ mol degraded/min/mg of protein.
ND, not detected.

Table IV. Inhibitor profiles of β-lactamases of strains listed in tables II and III

Bacterial strain	IC_{50} (μM)						
	pcmb	imipenem	latamoxef	cefoxitin	clavulanic acid	sulbactam	cloxacillin
B. fragilis R106	10	<0·1	<0·1	<0·1	<0·1	<0·1	10
B. fragilis R130	>100	<0·1	<0·1	<0·1	<0·1	1	100
B. fragilis R249	10	>100	>100	>100	>100	>100	100
B. fragilis R251	100	>100	>100	>100	>100	>100	100
B. fragilis R186	>100	>100	>100	>100	>100	>100	>100
B. fragilis R240	100	>100	>100	>100	>100	>100	>100
B. buccae R147	>100	<0·1	<0·1	<0·1	<0·1	1	>100
B. ovatus R137	>100	<0·1	<0·1	<0·1	<0·1	<0·1	100
B. ovatus R111	10	<0·1	<0·1	<0·1	<0·1	<0·1	100
B. bivius R160	>100	<0·1	<0·1	<0·1	<0·1	1	>100
R. distasonis R118	>100	<0·1	<0·1	<0·1	1	10	>100
B. fragilis R208	>100	<0·1	100	1	10	10	>100
B. fragilis R141	>100	<0·1	<0·1	<0·1	<0·1	<0·1	100
B. bivius R143	>100	<0·1	100	1	<0·1	10	>100
B. fragilis R126	>100	<0·1	1	<0·1	<0·1	<0·1	100
B. ovatus R215	>100	<0·1	10	1	1	1	100
B. fragilis R152	>100	<0·1	<0·1	<0·1	<0·1	1	100
B. ovatus R112	100	<0·1	<0·1	<0·1	<0·1	<0·1	100
B. fragilis R226	1	<0·1	<0·1	<0·1	<0·1	<0·1	10
B. fragilis R216	1	<0·1	<0·1	<0·1	<0·1	<0·1	1
B. ovatus R102	100	<0·1	<0·1	<0·1	<0·1	>100	>100
B. fragilis R212	>100	<0·1	100	1	1	1	>100
B. fragilis R134	>100	<0·1	<0·1	1	<0·1	<0·1	100
B. thetaiotaomicron R233	>100	<0·1	100	1	1	10	>100

IC_{50}, lowest concentration of inhibitor producing >50% inhibition; pcmb, *p*-chloromercuribenzoate.

HPLC

Figs. 1–4 show the rates of hydrolysis of benzylpenicillin (in the presence of clavulanic acid), cefoxitin, latamoxef and imipenem for those strains in which breakdown was detected by HPLC. In the presence of clavulanic acid, benzylpenicillin was rapidly degraded by enzymes from *B. fragilis* strains R186, R212, R240, R249 and R251 and *B. thetaiotaomicron* R233; enzymes from *B. bivius* R143 and *B. ovatus* R215 hydrolysed penicillin more slowly (fig. 1). All these strains, with the exception of *B. bivius* R143, also broke down cefoxitin and latamoxef (figs. 2 and 3); latamoxef was hydrolysed more rapidly than cefoxitin in all cases. Only four of the test strains, R186, R240, R249 and R251, were active against imipenem; all hydrolysed the antibiotic rapidly, but a small amount of the compound was still detectable after overnight incubation (fig. 4).

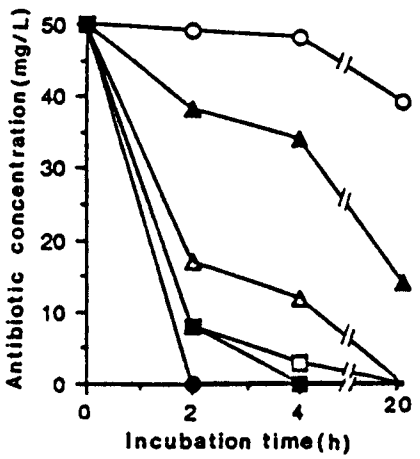

Fig. 1. Hydrolysis of benzylpenicillin in the presence of clavulanic acid 4 mg/L by enzyme extracts from *Bacteroides* strains; —○—, control; —●—, R212, R233, R240, R251; —△—, R215; —□—, R249; —■—, R186; —▲—, R143.

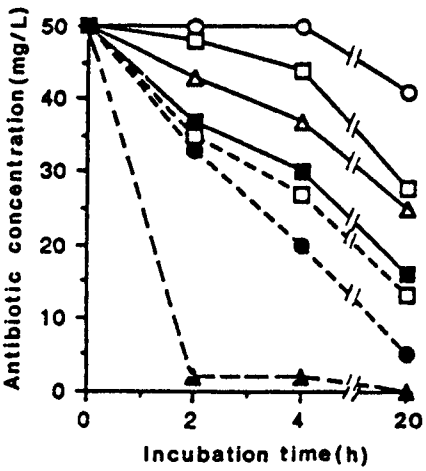

Fig. 2. Hydrolysis of cefoxitin by enzyme extracts from *Bacteroides* strains; —○—, control; --▲--, R212, R233; —△—, R215; —□—, R249; —■—, R186; --□--, R240; --●--, R251.

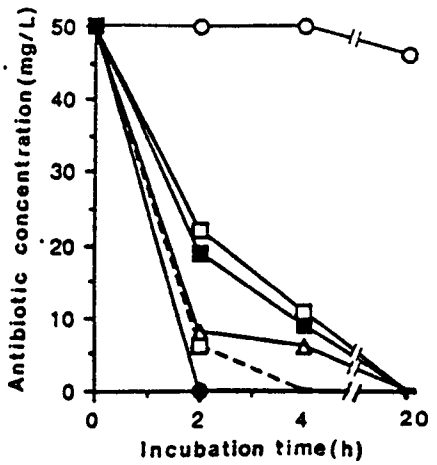

Fig. 3. Hydrolysis of latamoxef by enzyme extracts from *Bacteroides* strains; —○—, control; —●—, R212, R233, R251; —△—, R215; —□—, R249; —■—, R186; --□--, R240.

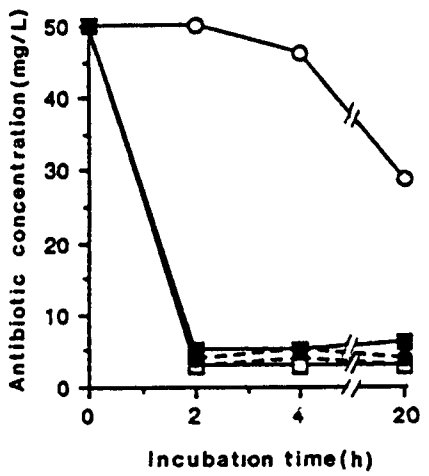

Fig. 4. Hydrolysis of imipenem by enzyme extracts from *Bacteroides* strains; —○—, control; —□—, R249; —■—, R186; --□--, R240; --●--, R251.

Discussion

Some unusual properties of the β-lactamases of *Bacteroides* spp. have been revealed in previous studies,[3,4,7] but the characterisation and classification of these enzymes remain ill-defined.

In the present study of 116 clinically significant isolates of *Bacteroides*, 24 produced levels of cephalosporinase activity greater than the basal level typical of most *Bacteroides* strains, and two-thirds of these also exhibited the capacity to hydrolyse penicillinase as judged by the phenol red test.[9] Fourteen of these 24 strains were identified as *B. fragilis*, and six of the

seven strains that failed to give a positive result in the test for penicillinase activity belonged to this species. In contrast to a previous study of 78 clinical isolates of the *B. fragilis* group, in which all but one of 13 "high β-lactamase" producers were identified as *B. fragilis sensu stricto*,[4] seven isolates were identified as other members of the *B. fragilis* group, including five *B. ovatus* strains.

As in the earlier study,[4] imipenem and the combination of benzylpenicillin with clavulanic acid were much more active than cefoxitin, latamoxef or benzylpenicillin alone, as judged by conventional MIC titration. The overall level of resistance remained low, although there seems to have been a slight increase in the prevalence of resistance to cefoxitin and imipenem in the intervening 5 years: for nine strains (8%), the MIC of cefoxitin was ≥32 mg/L; five of these strains (4%) were also resistant to latamoxef (MIC ≥32 mg/L). A total of seven strains (6%) displayed reduced susceptibility to imipenem (MIC ≥2 mg/L). The proportions of strains in the previous study exhibiting this level of resistance were cefoxitin (4%), latamoxef (6%) and imipenem (4%). Among the present strains, seven (6%) were able to hydrolyse cefoxitin, latamoxef or imipenem; 4% of strains in the earlier study displayed this property.

The categorisation of the β-lactamases of *Bacteroides* spp. is problematic. Thus, enzymes classified on the basis of their differential ability to hydrolyse β-lactamase-stable compounds such as cefoxitin, latamoxef, imipenem and clavulanic acid often exhibit heterogeneous properties according to other measures of activity, such as inhibition profiles and specific enzyme activity.

Use of specific activity as a differential feature presents particular problems, because the conditions of growth of the organisms, as well as the methods used for preparation, concentration and storage of the enzyme extracts, may profoundly affect the results.[11] These factors were carefully standardised in the present investigation, but variability originating in methodological differences makes comparison with the results of other studies difficult.

Despite the problem of reconciling so many heterogeneous properties, certain tentative groupings can be perceived among the strains described in the present study and others previously reported (table V).[4,7]

In the first group are the enzymes produced by *B. fragilis* strains R186, R240, R249 and R251, which appear to have broadly similar characteristics: low specific activity; insusceptibility to inhibition by β-lactamase inhibitors, including clavulanic acid; relative resistance to imipenem; and the ability to degrade imipenem, latamoxef and, more slowly, cefoxitin. These properties are similar to those previously described in *B. fragilis* strains, 57, 97 and 119,[4,7] although the failure to detect iso-electric focusing bands in *B. fragilis* strains R240, R249 and R251 is anomalous.

The second group is represented by *B. fragilis* R212, *B. ovatus* R215 and *B. thetaiotaomicron* R233, all of which exhibited resistance to benzylpenicillin, cefoxitin and latamoxef and reduced susceptibility to imipenem; they also displayed intermediate, or high specific activity. Enzymes from these strains showed similar (but not identical) inhibition profiles and hydrolysed β-lactamase-stable compounds other than imipenem. These enzymes are clearly not identical, since their iso-electric points are diverse as would be expected of enzymes from these different species. The overall behaviour is similar to that of enzymes produced by *B. thetaiotaomicron* 0456 and *B. distasonis* R939, described previously.[7]

A third group, represented by two strains, *B. distasonis* R118 and *B. fragilis* R208, exhibited reduced susceptibility to cefoxitin, latamoxef and imipenem, but did not hydrolyse these antibiotics as judged by HPLC. The enzymes displayed variable inhibition profiles and intermediate levels of specific activity. Other strains have been described that appear to be resistant to β-lactamase-stable compounds although they do not hydrolyse them. Such strains seem to belong to a heterogeneous group which may possess other mechanisms of resistance[1,5] and these will be the subject of further study.

Most of the strains encountered in this, and a previous study,[4] retain a high degree of susceptibility to cefoxitin, latamoxef and imipenem. Generally, the enzymes are fully susceptible to β-lactamase inhibitors and clavulanic acid fully restores the activity of benzylpenicillin. These strains produce enzymes with a wide range of specific activities that have no hydrolytic activity against β-lactamase-stable compounds. These strains were identified as enhanced β-

Table V. Characteristics of four types of β-lactamases from 24 *Bacteroides* strains

Type	MIC (mg/L) of				Inhibitors of β-lactamase	Activity of benzylpenicillin restored by clavulanic acid	Hydrolysis of			
	benzyl penicillin	cefoxitin	latamoxef	imipenem			benzyl penicillin*	cefoxitin	latamoxef	imipenem
Type 1	4–16	16–32	0·5–4	1–4	Resistant	No	Yes	Yes (slow)	Yes	Yes
Type 2	>64	>32	>128	1–4	Intermediate	Partial	Yes	Yes	Yes	No
Type 3	>16	32	>8	2–4	Variable	Yes	No	No	No	No
Type 4	4–>64	4–32	0·5–32	≤1	Susceptible	Yes	No	No	No	No

* In the presence of clavulanic acid.

lactamase-producers by the nitrocefin screening procedure and it is probable that they are strains that produce greater than normal amounts of the cephalosporinase that most *B. fragilis* group strains are known to produce.

The enzyme produced by *B. bivius* R143 did not conform to any of the categories described above, notably by slowly hydrolysing benzylpenicillin in the presence of clavulanic acid. In other respects its behaviour was similar to that of the other *B. bivius* strain R160, although it was considerably less susceptible to inhibitors of β-lactamase activity.

The strains that we have examined were selected on the grounds of their involvement in some pathogenic process and most of the species encountered belonged to the *B. fragilis* group. Others have described β-lactamases with heterogeneous properties from non-*B. fragilis* group species[15] and it is clear that the β-lactamases of anaerobic gram-negative rods are much more variable than those of enterobacteria.[16, 17] Fortunately, the number of strains that exhibit enzymic or other mechanisms of resistance to β-lactam antibiotics appears presently to be quite small, but there is no guarantee that this situation will continue to prevail under the selective pressure of antibiotic usage.

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Extended abstract

OUTER MEMBRANE ANALYSIS OF B.FRAGILIS STRAINS WITH REDUCED SUSCEPTIBILITY TO IMIPENEM

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Imipenem is normally highly active against *B.fragilis* (MIC ≤0.12 mg/l), but a minority of strains (about 6%) display reduced susceptibility (MIC ≥2 mg/l). Some of these strains produce imipenem-hydrolysing metallo-β-lactamases[1] but in others the reduction in susceptibility does not appear to be associated with enzymatic activity[2]. To ascertain the role of other resistance factors, such as reduced antibiotic penetration into the cell, the outer membrane (OM) protein composition (which reflects the porin content) and OM lipopolysaccharide (LPS) (which contributes to bacterial hydrophobicity) were examined. The OM compositions of 8 *B.fragilis* strains with reduced susceptibility to imipenem, 4 of which produced metallo-β-lactamases (R186, R240, R251 and 119) and 4 which were not metallo-β-lactamase producers (R208, R212, 0423 and 2013E) were compared to 2 fully sensitive *B.fragilis* strains (R135 and NCTC 9344) by SDS-PAGE.

All strains possessed a major OM protein of molecular weight 44 kDa and other main bands of 62 kDa, 42 kDa, 38 kDa, 29 kDa and 22 kDa. The profiles of metallo-β-lactamase producing *B.fragilis* were distinctive with an extra band of 40 kDa (figure 1). An additional band of 51 kDa was seen in the profile of *B.fragilis* R186. Also, *B.fragilis* R240

and 119 possessed an extra 16 kDa protein. The LPS compositions of the fully sensitive *B.fragilis* strains and of those resistant strains that did not produce metallo-β-lactamase were similar, with a major component (band B) and additional bands of larger (band A) and smaller (band C) molecular size. LPS of band A was not present in strains that produced metallo-β-lactamases and band B was either absent or more diffuse in these strains.

B.fragilis strains with reduced susceptibility to imipenem, but which do not produce imipenem hydrolysing metallo-β-lactamases appeared to possess OM protein and LPS profiles that were similar to those of fully sensitive strains. As the OM represents a permeability barrier, these findings suggest that antibiotic penetration into the cell is not responsible for the increase in resistance of these strains. A reduction in the affinity of imipenem for the penicillin-binding proteins of these strains may account for their reduced sensitivity and this is being investigated. Suprisingly, the metallo-β-lactamase-producing *B.fragilis* strains showed OM protein and LPS profiles that differed from those of the non-metallo-β-lactamase producers. Their additional protein band of 40 kDa may represent metallo-β-lactamase. The distinctive LPS profile of the strains that produce metallo-β-lactamases may simply represent differences in O side chains indicating differences in serotype.

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Comparative Stability of Meropenem against β-Lactamases from *Bacteroides* spp.

Key Words
Meropenem
β-Lactamases
Bacteroides spp.

Abstract
The activity of meropenem was similar to that of imipenem against 88 clinical isolates of *Bacteroides* spp., including 28 non-*fragilis* strains. Four of the isolates were able to hydrolyse cephamycins or penems. Investigation of β-lactamases from these strains and 10 laboratory stock culture *Bacteroides* showed that meropenem and imipenem were degraded at a similar rate by cell-free preparations of these enzymes. Some of these strains were able to completely hydrolyse the carbapenems within 2 h. Despite this rapid hydrolysis, MICs of meropenem and imipenem for some of the strains remained within the susceptible range as judged by agar dilution titration.

Introduction

Meropenem is a new carbapenem with broad-spectrum activity and stability to most β-lactamases [1–4]. Most clinical isolates of *Bacteroides* spp. are unable to hydrolyse carbapenems or cephamycins, but potent β-lactamases that degrade cefoxitin and imipenem are occasionally encountered and are associated with a reduction in antibiotic susceptibility [5–8]. In this study, the in vitro activity of meropenem was compared with that of four other anti-anaerobe β-lactam agents against clinical isolates of *Bacteroides* spp. The comparative stability to enzymes from these and other strains capable of hydrolysing carbapenems and cephamycins was examined.

Materials and Methods

Eighty-eight clinically significant isolates of *Bacteroides* spp. were collected from specimens submitted to Nottingham Public Health Laboratory, and maintained in skimmed milk at −70 °C. They were identified by standard methods. Ten laboratory stock cultures of *Bacteroides* which have been shown to hydrolyse carba-

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penems or cephamycins in previous investigations [5–7] were also used. *B. fragilis* NCTC 9343 was used as a control strain.

Meropenem, imipenem and clavulanic acid were gifts from the manufacturers; standard pharmaceutical preparations of cefoxitin, piperacillin and benzylpenicillin were used. MICs were determined by the agar dilution method with brain heart infusion agar supplemented with yeast extract 5 g/l, haemin 5 mg/l and menadione 1 mg/l. Inocula of about 10^4 and 10^6 c.f.u./spot were delivered onto the agar surface with a multipoint inoculating device. Results were recorded after incubation for 48 h at 37 °C in an anaerobic cabinet.

Crude β-lactamase extracts were obtained by sonication of bacterial suspensions freshly prepared as described elsewhere [9]. For high-pressure liquid chromatography (HPLC) 0.2 ml of enzyme extract was mixed with 0.8 ml of antibiotic solution to give a final concentration of 50 mg/l and incubated at 37 °C. Samples were introduced into a Hypersil 5ODS HPLC column after 0, 2, 4 and 20 h. The HPLC conditions for imipenem, cefoxitin and penicillin have been previously described [7]; for meropenem the mobile phase was methanol: water (25 : 75) with spectrophotometric detection at 300 nm.

Table 1. Antibiotic susceptibilities of *Bacteroides fragilis* and non-*fragilis* strains to selected β-lactam antibiotics (inoculum 10^6 c.f.u./spot)

Antibiotic	MIC, mg/l		
	range	MIC_{50}	MIC_{90}
B. fragilis (n = 60)			
Meropenem	0.06–4	0.25	0.5
Imipenem	0.06–2	0.25	0.5
Piperacillin	0.5–> 128	4	64
Cefoxitin	⩽2–128	8	16
Penicillin with clavulanic acid[a]	⩽0.015–16	0.25	1
Other *Bacteroides* spp. (n = 28)			
Meropenem	0.25–4	0.5	0.5
Imipenem	0.12–4	0.5	1
Piperacillin	4–> 128	16	64
Cefoxitin	⩽2–> 128	16	16
Penicillin with clavulanic acid[a]	⩽0.015–16	0.25	4

[a] Clavulanic acid: 4 mg/l.

Results and Discussion

Of the 88 clinical isolates of *Bacteroides*, 60 were identified as *B. fragilis*, 13 as *B. distasonis*, 8 as *B. ovatus*, 4 as *B. thetaiotaomicron* and 3 as *B. vulgatus*. Overall, meropenem and imipenem were the most active of the antibiotics tested, with MIC_{90}s of 0.5–1 mg/l (table 1). The combination of benzylpenicillin and clavulanic acid showed activity similar to that of the carbapenems against *B. fragilis* isolates, but less activity against the non-*fragilis* strains (MIC_{90} of 4 mg/l). Cefoxitin and piperacillin were less active than the carbapenems or clavulanate-potentiated penicillin against these strains. Inoculum effects were small or absent in all cases. These findings are in broad agreement with other studies [1–4].

Four of the clinical isolates (*B. fragilis* R186 and R212, *B. thetaiotaomicron* R233 and *B. ovatus* R215), representing 5% of the strains studied, showed reduced susceptibility to the carbapenems or cefoxitin and possessed β-lactamases which hydrolysed these compounds.

Investigation of these 4 isolates together with 10 laboratory stock culture strains possessing β-lactamases capable of hydrolysing carbapenems or cephamycins showed that imipenem was 2- to 4-fold more active than meropenem for 7 of these strains (table 2). HPLC analysis revealed that 6 of these 7 isolates (and 1 other, *B. fragilis* 119, which was resistant to both imipenem and meropenem) were able to hydrolyse meropenem completely within 2 h. With 1 of these strains (*B. fragilis* R186), complete hydrolysis of imipenem was not observed even after incubation for 20 h (table 3). These penem-degrading β-lactamases are metallo-enzymes [10] and, as expected, clavulanic acid did not protect ben-

Table 2. Antibiotic susceptibilities of 14 *Bacteroides* strains with β-lactamases capable of hydrolysing carbapenems or cephamycins (inoculum 10^6 c.f.u./spot)

Strain	MIC, mg/l			
	mero-penem	imi-penem	cefo-xitin	peni-cillin[a]
B. fragilis R240	8	2	32	4
B. fragilis R251	4	2	32	2
B. fragilis R249	2	0.5	16	4
B. fragilis R186	4	2	32	8
B. fragilis 97	2	0.5	16	8
B. fragilis 57	> 8	8	32	> 8
B. fragilis 119	> 8	> 8	64	> 8
B. fragilis 107	0.5	0.5	32	1
B. fragilis 0423	0.5	1	32	1
B. fragilis R212	2	2	128	4
B. thetaiotaomicron R233	4	4	> 128	16
B. thetaiotaomicron O456	4	2	> 64	4
B. ovatus R215	1	1	64	0.5
B. distasonis R939	0.5	1	32	> 8
B. fragilis NCTC9343[b]	0.12	0.12	4	0.12

[a] Tested in the presence of 4 mg/l clavulanic acid.
[b] Control strain.

Table 3. Degradation of penicillin in the presence of clavulanic acid (4 mg/l), cefoxitin, imipenem and meropenem by β-lactamases from 14 strains of *Bacteroides* spp.

Strain	Antibiotic remaining, %[a]											
	meropenem			imipenem			cefoxitin			penicillin		
	2 h	4 h	20 h	2 h	4 h	20 h	2 h	4 h	20 h	2 h	4 h	20 h
B. fragilis R240	0	0	0	0	0	0	68	49	31	0	0	0
B. fragilis R251	0	0	0	0	0	0	64	41	11	0	0	0
B. fragilis R249	0	0	0	8	0	0	81	72	58	0	0	0
B. fragilis R186	0	0	0	20	15	6	81	72	64	0	0	0
B. fragilis 97	0	0	0	6	0	0	75	68	58	0	0	0
B. fragilis 57	0	0	0	0	0	0	21	9	0	0	0	0
B. fragilis 119	0	0	0	0	0	0	28	11	0	0	0	0
B. fragilis 107	100	100	62	92	87	16	89	87	83	78	71	0
B. fragilis 0423	97	97	24	84	78	10	83	81	72	0	0	0
B. fragilis R212	98	98	41	92	98	61	0	0	0	0	0	0
B. thetaiotaomicron R233	98	98	59	88	95	39	0	0	0	0	0	0
B. thetaiotaomicron 0456	100	100	27	94	97	4	30	23	0	0	0	0
B. ovatus R215	98	98	8	92	98	61	79	65	65	4	0	0
B. distasonis R939	100	100	22	94	89	4	83	79	67	4	0	0
B. fragilis NCTC9343[b]	100	100	95	100	100	95	100	98	95	100	95	90

[a] Percentage relative to the organism-free control value after incubation at 37 °C for the time indicated.
[b] Control strain.

zylpenicillin from hydrolysis; as reported previously [11, 12] cefoxitin was more stable than carbapenem compounds to these enzymes.

Despite the rapid hydrolysis of meropenem and imipenem by crude cell-free extracts of certain strains of *Bacteroides* spp., MICs of these compounds were not always raised above 4 mg/l. These isolates would be regarded as susceptible according to conventional criteria [13], but the MICs of carbapenems for these strains were about 10-fold higher than those seen with fully sensitive *Bacteroides* strains.

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